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Dental Stem Cells and Leukocyte- and Platelet-Rich Fibrin as Candidate Therapies for Joint Repair

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Success is not the absence of failure;

it's the persistence through failure

- Aisha Tyler

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List of Abbreviations

ADAM	A disintegrin and metalloproteinase
3D	Three-dimensional
3-T	3-Tesla
AAEP	American Association of Equine Practitioners
ABMSCs	Alveolar bone-derived MSCs
ACI	Autologous chondrocyte implantation
ACL	Anterior cruciate ligament
ACPCs	Articular cartilage progenitor cells
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
AT-MSCs	Adipose tissue-derived mesenchymal stem cells
BLI	Bioluminescence imaging
BM-MNCs	Bone marrow mononuclear cells
BM-MSCs	Bone marrow-derived mesenchymal stem cells
BMPs	Bone morphogenetic proteins
BMS	Bone marrow stimulation
CBCT	Cone beam computed tomography
CM	Conditioned medium
CT	Computed tomography
CTA	Computed tomography arthrography
CTGF	Connective tissue growth factor
DAB	3,3'-Diaminobenzidine

DAPI	4’,6-Diamidino-2-phenylindole
DePDL	Stem cells from deciduous periodontal ligament
DFPCs	Dental follicle precursor cells
dGEMRIC	Delayed gadolinium-enhanced MRI of cartilage
DMEM	Dulbecco's modified Eagle’s medium
DMMB	Dimethyl-methylene blue
DPSC CM	Conditioned medium of DPSCs
DPSCs	Dental pulp stem cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESCs	Embryonic stem cells
ETCs	Embryonic tendon cells
EVs	Extracellular vesicles
EYA	Eye absent homologue
FABP-4	Fatty acid binding protein-4
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FLI	Fluorescence imaging
GAGs	Glycosaminoglycans
GDF	Growth/differentiation factor
GelMA	Methacrylated gelatin

GMSCs	Gingival MSCs
GRO	Growth regulated oncogene
H&E	Haematoxylin and eosin
HA	Hyaluronic acid
HHGS	Histological-Histochemical Grading System
IA	Intra-articular
ICC	Immunocytochemistry
ICRS	International Cartilage Repair Society
IDO	Indoleamine 2,3-dioxygenase
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
iMACs	Immature murine articular chondrocytes
iNOS	Inducible nitric oxide synthase
iPSCs	Induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
L-PRF	Leukocyte- and platelet-rich fibrin
L-PRF CM	L-PRF conditioned medium
L-PRF ex	L-PRF exudate
MACI	Matrix-induced autologous chondrocyte implantation
MCP	Monocyte chemoattractant protein
MDSCs	Muscle-derived stem cells
MFC	Medial femoral condyle
MMP	Matrix metalloproteinase

MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
MSDs	Musculoskeletal disorders
MTC	Medial tibial condyle
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
ORO	Oil red O
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem cells
pDSCs	Puppy deciduous teeth stem cells
PEGDMA	Poly(ethylene) glycol dimethacrylate
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PGs	Proteoglycans
PLGA	Poly(lactic-co-glycolic acid)
PLLA/PEG	Poly-l-lactic acid/polyethylene glycol
P-PRF	Pure platelet-rich fibrin
PRF	Platelet-rich fibrin
PRP	Platelet rich plasma
RA	Rheumatoid arthritis
RER	Rough endoplasmic reticulum
RT	Room temperature

RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
S.E.M.	Standard error of mean
SCAPs	Stem cells from the apical papilla
SHEDs	Stem cells from the pulp of exfoliated deciduous teeth
SHG	Second harmonic generation
Six	Sineoculis homeobox homolog
SPIO	Superparamagnetic iron oxide
SSCs	Skeletal stem cells
TBS	Tris buffered saline
TCZ	Tocilizumab
TDSCs	Tendon-derived stem cells
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-beta
TGPCs	Tooth germ progenitor cells
TIMP	Tissue inhibitors of metalloproteinase
TMJ	Temporomandibular joint
TMJOA	Temporomandibular joint osteoarthritis
TNF-α	Tumour necrosis factor-alpha
TSPCs	Tendon stem/progenitor cells
UCB-MSCs	Umbilical cord blood-derived mesenchymal stem cells
UCMSCs	Umbilical cord mesenchymal stem cells
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZOL	Ziekenhuis Oost-Limburg

αMEM	Minimal essential medium, alpha modification

Chapter 1: General Introduction and Aims

Partially based on;

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1.1. Musculoskeletal Disorders and the Synovial Joint

Musculoskeletal disorders (MSDs) include more than 150 different pathologies. They can vary from short-term injuries to chronic disorders associated with long-term discomfort and disability. According to the World Health Organization (WHO), musculoskeletal injuries are the most common cause of severe long-term pain and physical disability, and affect hundreds of millions of people around the world. In addition, they are often leading to significant mental health weakening and increased risk of other chronic conditions. Although the prevalence of major musculoskeletal conditions increases with age, they are not only affecting older people (1). Moreover, the increasing popularity of sports caused a widespread of MSDs. They occur to joints, but also to the surrounding soft tissues that contribute to their movement; they can affect but are not limited to muscles, bones, joints, cartilage, ligaments, and tendons (1).

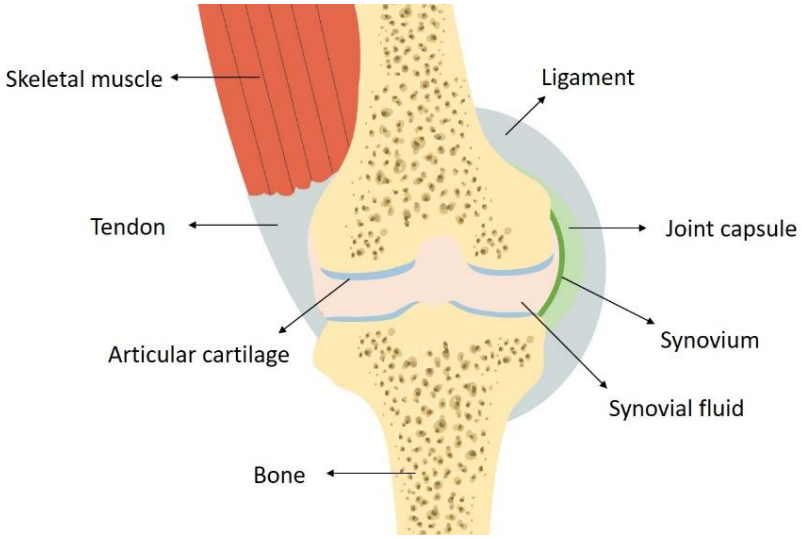

Figure 1.1. Structure of the human synovial joint. The synovial fluid, produced by the synovium or synovial membrane, together with the articular cartilage, which covers the ends of bone, allow the frictionless movement of the bones over one another. The joint capsule is a fibrous tissue surrounding the joint. The ligaments surrounding the joint prevent over-flexion or -extension. Tendons stabilize joints, attach skeletal muscles to bones and transform the contraction of the muscle into movement of the joint. *This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.*

Joints or articulations are the connections between bones and can be divided in three groups based upon the degree of movement they allow: synarthrosis joints allow little or almost no movement, amphiarthrosis joints, such as intervertebral discs, permit partial movement and the third group consists of synovial joints or diarthrosis (2). The synovial joint (Figure 1.1) is the most common joint in the human body and allows free but restricted angular movement of articulating bones. It consists of articular cartilage, synovium, synovial fluid, perichondrium, and subchondral bone (Figure 1.1) (3). The lubricated surface of the articular cartilage covers the ends of bones, allowing the smooth movement of bones at the joint site (2, 4). Stability of joints is ensured by soft tissue structures, such as tendons or articular ligaments (5).

Degenerative diseases and overloading of the joint may eventually result in irreversible damage to tissues from the joint, comprising articular cartilage and tendons. Cartilage injuries are very common, and are especially highly prevalent in subjects with knee osteoarthritis (OA) (6), one of the most common and debilitating MSDs (1). In addition, 30% of all consultations related to MSDs are reported to be tendon-related (7). There is a growth in the prevalence of OA and tendinopathies and an increase in costs, leading to a substantial economic impact of both conditions. Both pathologies involve tissues that are characterized by a low intrinsic regenerative capacity due to the low vascularity and cell content. Furthermore, current treatment options are not able to provide full and stable recovery of the damaged tissue (8-10). Moreover, despite significantly improved knowledge and understanding of the pathophysiology of both diseases, there is still an increasing need for the development of new treatment strategies for OA, cartilage defects and tendon injuries (11).

1.2. Cartilage Defects and Osteoarthritis

1.2.1. Pathophysiology and Current Treatments

Articular cartilage covers the ends of bone, due to its slightly compressible nature and lubricated surface, it provides the joint with shock absorption and lubrication (4, 8). Hyaline cartilage is comprised of 95% extracellular matrix (ECM) (dry weight) and only 5% of sparsely distributed chondrocytes (12). This matrix primarily consists of type II collagen and proteoglycans (PGs). Negatively charged

glycoproteins are able to attract water, allowing cartilage to resist compressive forces (13). Despite the fact that chondrocytes only make up about 5% of hyaline cartilage tissue, they are integral for cartilage function and homeostasis (13). These cells are of mesenchymal origin and are responsible for synthesizing cartilage ECM (12). Hyaline cartilage is an avascular tissue which, in part, explains the limited regeneration following injury. The lack of vasculature makes it difficult for progenitor cells to be recruited to the site of injury and hinders the supply of nutrients necessary for tissue repair (8, 9).

Cartilage loss can occur as a consequence of traumatic injury, leading to focal defects, or through chronic degeneration. Both partial thickness or full thickness cartilage defects occur (14). Since full thickness lesions extend into the subchondral bone, they have access to bone marrow cells and therefore have a higher probability of spontaneous regeneration than partial thickness lesions, which only involve the avascular cartilage tissue (14). Eventually cartilage defects will lead to activity-related pain, swelling and decreased mobility and will frequently progress to OA (8, 15). In the United States alone, over 27 million adults suffer from OA, while in Europe it is estimated to affect more than 40 million people, leading to a substantive clinical and financial burden (16-18).

OA is the most common form of arthritis and affects the large weight bearing joints such as hip and knee, but also smaller joints such as digits. Pathological changes seen in OA joints include progressive destruction of articular cartilage, thickening of the subchondral bone, formation of osteophytes, variable degrees of inflammation of the synovium and degeneration of ligaments or tendons and menisci of the knee (19).

For long times, OA has been considered as a disease of cartilage degradation. However, improved understanding of the pathophysiology unravelled that the disease affects the entire joint, in which matrix proteases play a crucial role. Under normal conditions, cartilage matrix is exposed to continuous ongoing remodelling in which degenerative and synthetic enzymatic activities are balanced. However, in OA cartilage, matrix degrading enzymes are upregulated, which results in shifting the balance towards degeneration, release of pro-inflammatory cytokines, and eventually evolves in loss of collagen and PGs. In addition, the subchondral bone, menisci and ligaments and the synovium have been described to play key

roles in OA pathogenesis, and are associated to the pro-inflammatory status of the entire joint as well as the systemic inflammation (20-22). Different cellular changes and biomechanical stress lead to secondary OA features, including subchondral bone remodelling, the development of osteophytes, the formation of bone marrow injuries and synovial changes (20).

There are currently no treatments available to effectively heal cartilage defects. When cartilage defects develop into OA, the condition can only be managed by a multidisciplinary approach including pharmacotherapy, physiotherapy or joint replacement surgery (23). However, several surgical interventions can be performed in order to prevent progression towards OA (8). Current techniques include: arthroscopic lavage and debridement, microfracture induction and autologous chondrocyte implantation (ACI) (11). Although these techniques have been proposed to restore normal joint function and minimize further degeneration, they often do not offer a long-term clinical solution. There is a clinical need to develop regenerative medicine approaches to permanently restore articular cartilage (11).

Within cellular regenerative therapeutic applications, ACI, the use of mesenchymal stem cells (MSCs) and platelet concentrates are of particular interest. ACI consists of a three-step procedure involving harvesting, culturing and re-implantation of autologous chondrocytes into the defect. Nevertheless, the technique can be associated with several drawbacks, such as an increased risk for complications, iatrogenic damage, the need of two surgeries, and low integration of the chondrocyte implantation (24, 25). For these reasons, other regenerative approaches, including platelet derivatives and stem cell-based therapies, have experienced substantial research attention.

1.3. Stem-Cell Based Approaches for Cartilage Regeneration and Osteoarthritis

1.3.1. Current *In Vitro* Evidence of Chondrogenic Differentiation of Stem Cells

For stem cell-based cartilage regeneration, MSCs are of particular interest because, in comparison to chondrocytes, they have high availability, and are both

easy isolated and expanded (26). In addition, their *in vitro* chondrogenic differentiation potential has been demonstrated (27). More recently, *in vitro* studies on induced pluripotent stem cells (iPSCs) indicated promising results for their use in cartilage repair (28, 29). However, a number of challenges need to be overcome and further optimization is still needed before both stem cell types can be used as a safe and effective therapeutic option for promoting cartilage repair (8, 30-33).

Mesenchymal stem cells

Adult MSCs were first identified in bone marrow (34, 35), but afterwards, other MSC niches have been discovered in both adult and foetal tissues, including adipose tissue (36), placenta (37), umbilical cord (38), dental pulp (39), peripheral blood (40) and in the synovial membrane (41). As defined by the International Society for Cellular Therapy (ISCT), MSCs must be able to differentiate into chondrocytes under specific *in vitro* conditions (42). In addition, MSCs possess additional properties making them a suitable cell source for cartilage regeneration. High cell numbers can be produced and the immunomodulatory characteristics of MSCs allow for their allogeneic use (43).

Pellet and monolayer cultures are the two main culture systems that have been developed to study *in vitro* chondrogenic differentiation. The three-dimensional (3D) pellet system is the most representative *in vitro* model for the condensation of mesenchymal cells that is observed during the initiation phase of chondrogenesis in the process of endochondral ossification (44, 45). Moreover, co-cultures with chondrocytes in both 2D and 3D culture systems could push MSCs towards the chondrogenic lineage (46-48) and growth factors such as insulin-like growth factor (IGF) (49), and members of the fibroblast growth factor (FGF) (50) and transforming growth factor-beta (TGF-β) (51-53) families, can be added to the differentiation medium to enhance chondrogenic differentiation. Additionally, the chondrogenic differentiation potential of MSCs and the production of ECM proteins can also be stimulated by combining MSCs and biomaterials in 3D scaffolds (54-61) or by manipulating the oxygen tension (62).

In vitro studies mainly focus on bone marrow-derived mesenchymal stem cells (BM-MSCs), followed by MSCs derived from adipose tissue and synovial membrane because of their easy isolation and close proximity to cartilage and

joints, respectively (63). A correlation between the chondrogenic potential of MSCs and their tissue source has been suggested. BM-MSCs showed a superior chondrogenic differentiation capacity compared to MSCs from other origins (64-66). These differences might be explained by variations in gene expression and pathway activation (67). Therefore, an adapted differentiation protocol for other MSC sources could compensate for lower chondrogenic differentiation capacities (67, 68).

Despite their promising chondrogenic potential *in vitro*, several challenges are linked to the use of MSCs in cartilage regeneration. The most common issue is terminal differentiation towards hypertrophic cells (45). Moreover, mineralization and vascularization have also been reported after transplantation (44, 69). In addition, cartilage tissue derived from *in vitro* differentiated MSCs resembles fibrocartilage with inferior mechanical properties and healing capacity (30). Another limitation is the inter- and intra-donor heterogeneity of MSCs which could influence chondrogenic differentiation potential of cells (70), depending on comorbidities, tissue source and culture methods (33).

Induced pluripotent stem cells

Part of the issues associated with MSCs can be circumvented by using iPSCs. These cells are transformed from fibroblasts to pluripotent stem cells by retroviral transduction with the transcription factors Oct3/4, Sox2, Klf4, and c-Myc, the so-called Yamanaka factors, which makes them an ideal patient-specific unlimited cell source for autologous tissue regeneration (71). Promising *in vitro* results have already been demonstrated in the cartilage engineering field for iPSCs generated from various cell types (28, 29, 31, 72, 73). Nevertheless, Guzzo *et al.* stressed the influence of cell type origin on their chondrogenic capacity, where superior properties could be assigned to iPSCs from chondrogenic origin (74), which may be due to the preservation of the epigenetic memory (75).

Analogous to MSCs, indirect co-cultures of iPSCs with primary chondrocytes could directly induce the formation of chondrocytes (28). Furthermore, iPSCs could be committed to the chondrogenic lineage in high-density pellet culture systems, enhanced by the addition of growth factors from the TGF-β superfamily. Nevertheless, the resulting cartilage is a heterogeneous combination of hypertrophic-, articular- and fibrocartilage (76). This heterogeneity could be

reduced by first differentiating iPSCs towards an intermediate cell population, such as MSCs (76, 77) or embryonic cell types (31, 73, 78). An alternative approach to further enhance the chondrogenic potential is seeding iPSCs into scaffolds, such as nanofibrous scaffolds (79).

Although iPSCs express higher proliferation rates (80) and similar or superior chondrogenic differentiation potential (32, 72) compared to MSCs, other limitations remain associated with these stem cells. Patient-specific autologous iPSC generation and transplantation is very expensive and would therefore not be a therapeutic option for all patients. Allogeneic therapy would be more attractive, but immune rejection cannot be excluded (81). Analogous to MSCs, it remains uncertain whether the regenerated cartilage induced by iPSCs preserves the mechanical and functional properties of native articular cartilage. Furthermore, also for iPSCs, the presence of hypertrophic signals under *in vitro* conditions, even though to a lesser extent than for MSCs, might indicate the formation of low-quality cartilage tissue by iPSCs (31, 32). Safety issue is the most important concern that hampers their general use (82). The potential reactivation of pluripotency in iPSCs or iPSC-derived chondrocytes should be addressed (83). Moreover, when using retrovirally transduced iPSCs, where the retroviral gene is integrated in the host, a higher risk for teratoma formation in cell transplants is reported (84). Therefore, adequate phenotyping of (fully) chondrogenic committed iPSCs is needed before transplantation of cells in (pre)clinical use.

Other Sources

In addition to the above mentioned stem cell sources, also articular cartilage progenitor cells (ACPCs) might present a potential favourable stem cell source (85). Though articular cartilage is unable to heal spontaneously, a population of stem or progenitor cells from articular cartilage was identified. ACPCs can be found in the surface zone of articular cartilage and are responsible for cartilage homeostasis (85, 86). They can offer multiple advantages over BM-MSCs, since they undergo a more stable chondrogenesis and have been shown to be resistant to hypertrophic chondrogenic differentiation (87). Nevertheless, their use is hampered by drawbacks such as donor site morbidity or limited availability.

Also skeletal stem cells (SSCs) may have potential and therapeutic function. They reside at the growth plate and the periosteum and can differentiate towards bone, cartilage, and bone marrow (88, 89). While their function in bone growth and homeostasis of the skeleton is recognised, the precise definition of SCCs requires a common consensus, mainly because of the distorted similar use of terms of SCCs and MSCs (89). This is mainly due to the fact that MSCs have been used to describe any cell type with stem cell properties. *Bona fide* SSCs are bone-resident stromal stem cells. Similarly, any other connective tissue contains a comparable, but specific stem cell type (89).

1.3.2. Mechanisms of Action of Stem Cell-based Therapies for Cartilage Regeneration and Osteoarthritis

Stem cell-based therapies were initially developed as a cell replacement therapy due to the chondrogenic differentiation potential of stem cells (31, 32, 61, 90, 91). Moreover, differentiated MSCs, embryonic stem cells (ESCs) and iPSCs secrete PGs and collagen type II (31, 92-95) which are essential components of cartilage tissue. However, it has been shown that upon intra-articular (IA) transplantation, MSCs induce cartilage replacement, but the principle source of repair tissue is derived from endogenous cells (96). Therefore, it is postulated that the paracrine effect of the transplanted cells on the damaged host environment is mainly responsible for stimulating cartilage regeneration (Figure 1.2). MSCs that were exposed to tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), were shown to upregulate the expression of several growth factors, anti-inflammatory mediators (*vide infra*) and anti-catabolic factors ultimately leading to (stem) cell-mediated cartilage regeneration (Reviewed in (97, 98)). The main growth factors associated with cartilage regeneration that are secreted by MSCs belong to the TGF-β superfamily (99). Moreover, adipose tissue-derived mesenchymal stem cells (AT-MSCs) were demonstrated to diminish matrix metalloproteinase (MMP)-13 expression upon transplantation, potentially counteracting collagen degeneration in pathological cartilage (100). In addition to the paracrine effect of soluble factors, extracellular vesicles (EVs), released by MSCs, have been shown to influence cartilage regeneration (Figure 1.2). Reports on stem cell EV-mediated cartilage repair are scarce. However, studies showed that MSC-EVs promoted the formation of new cartilage and the deposition of

collagen type II and glycosaminoglycans (GAGs) (101). Additionally, EVs from MSCs that overexpressed miR-140-5p stimulated chondrocyte migration and proliferation (102). Moreover, it was recently reported that BM-MSCs secrete hyaluronic acid (HA)-coated EVs (103), which may allow MSC homing to cartilage defects in receptor-mediated way via CD44. Although stem cell-EVs have shown beneficial effects in cartilage repair, it should be noted that EVs may also have damaging effects in arthritis (104).

Furthermore, it has been demonstrated that MSCs possess several immunomodulatory properties (Figure 1.2) (105). Given the immune component underlying cartilage degeneration, modulating the immune response might contribute to reducing cartilage loss in diseases where an uncontrolled immune response is detrimental (106, 107). In OA, in addition to cartilage destruction, substantial synovial inflammation is reported. The secretome of MSCs, rich in anti-inflammatory cytokines, was demonstrated to balance the immune response in the synovium through decreasing the production of inflammatory mediators in OA explants (108).

First, MSCs are reported to inhibit proliferation of both $CD4^+$ and $CD8^+$ T cells in a dose-dependent way. Moreover, a shift from Th1 to Th2 inflammatory cells combined with a change in inflammatory cytokine profile was induced by MSCs. Also the formation of regulatory T cells *in vitro* and *in vivo* is assisted via MSCs (109). BM-MSCs, for example, have been shown to suppress T-cell proliferation (110, 111) and to induce T-cell apoptosis (112). The resulting debris stimulated phagocytes to produce TGF-β which increased the number of regulatory T cells (112). Moreover, T-cell proliferation was inhibited by BM-MSCs via production of prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO), which are two main effectors of MSC-mediated immune-suppression (108, 113, 114). PGE2 and IDO are also crucial in MSC-mediated inhibition of NK cell activation (115). MSCs are reported to inhibit NK cell proliferation and impair cytotoxic activity and NK cell cytokine production (109).

The proliferation, activation, maturation and antigen presentation of dendritic cells was also inhibited by MSC subtypes (116-120) and macrophage/microglia polarization was shifted towards an anti-inflammatory phenotype after exposure to MSCs, their secretome, or EVs (118-123) (reviewed by Weiss *et al.* (109)). IL-

1 Receptor Antagonist, secreted by MSCs, is described to promote the polarization of macrophages towards a more anti-inflammatory phenotype, which in turn secrete high levels of IL-10 and show decreased expression of TNF-a and IL-17. Moreover, IL-10 prevents monocyte differentiation into dendritic cells and shifts monocytes towards an anti-inflammatory, IL-10-secreting subtype contributing to a positive-feedback loop (109). Apart from IL-10, MCS-stimulated monocytes express high levels of MHC class II, CD45R, and CD11b and seem to be able to suppress T-cell activity. Regardless from this cytokine-regulated shift in monocyte polarization, a cytokine-independent pathway has also been exposed, by which phagocytosis of MSCs caused monocytes to shift into a type 2 anti-inflammatory phenotype (109). Concerning dendritic cells, they seem to be less active in $CD4^+$ T cell proliferation and to present an MHC class II-peptide complex. Moreover, in attendance of MSCs, type 1 dendritic cells secrete less TNF-α, while type 2 dendritic cells increased IL-10 secretion (109).

Additionally, MSCs were able to modulate the B cell response by paracrine actions (124, 125). MSCs are reported to decrease plasmablast formation of B cells as well as increasing the number of regulatory B cells (109).

Next to MSCs, iPSC- or ESC-derived MSCs could also inhibit lymphocyte proliferation and function (126-129) and NK cell function (128).

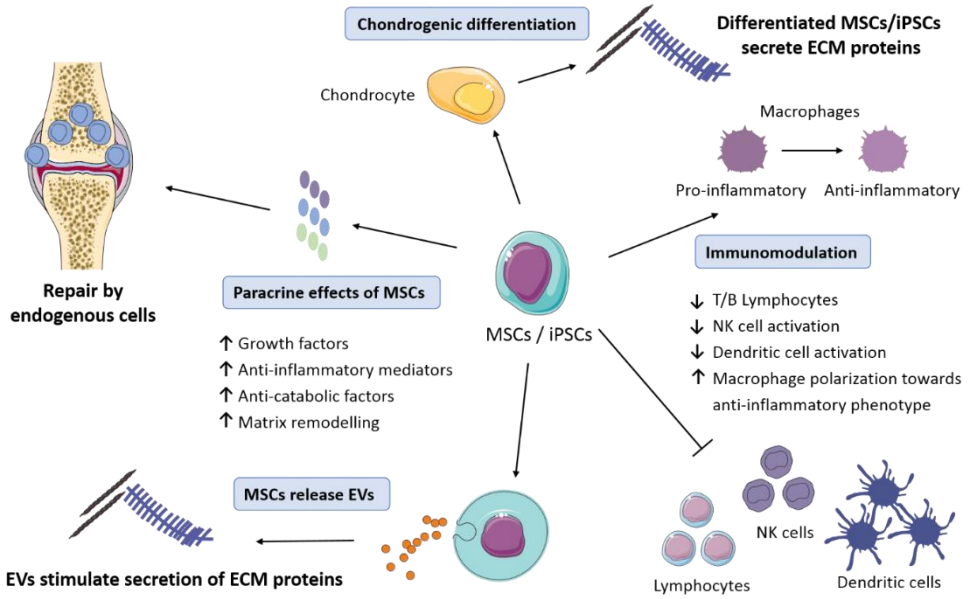

Figure 1.2. Mechanisms of action of stem cell-based therapies in cartilage regeneration and osteoarthritis (OA). First, stem cells could be applied as cell replacement therapy because of their chondrogenic differentiation potential. Differentiated mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) secrete proteoglycans (PGs) and collagen type II. Secondly, it is suggested that the tissue is regenerated by endogenous cells under the influence of paracrine factors secreted by stem cells. Extracellular vesicles (EVs) contribute to stem cell-mediated cartilage regeneration by promoting the formation of new cartilage and the deposition of collagen type II and glycosaminoglycans (GAGs). Finally, immunomodulatory effects are also observed. *This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.*

1.3.3. Dental Pulp Stem Cells for Cartilage Regeneration and Osteoarthritis

As previously mentioned, MSCs can be found in the stroma of any adult organ of the human body. Still, it remains to be elucidated which is the most suitable source of MSCs for the treatment of cartilage injuries or OA-associated lesions. Several subpopulations of MSCs can be distinguished within the human tooth and surrounding tissues (Figure 1.3), such as dental pulp stem cells (DPSCs) (130), stem cells from the apical papilla (SCAPs) (131), periodontal ligament stem cells (PDLSCs) (132), dental follicle precursor cells (DFPCs) (133) and tooth germ

progenitor cells (TGPCs) (134). Additionally, the gingiva contains other MSC subtypes; gingival MSCs (GMSCs) and the alveolar bone comprises alveolar bone-derived MSCs (ABMSCs) (135, 136). In addition, stem cells from the pulp of human exfoliated deciduous teeth (SHEDs) and deciduous periodontal ligament (DePDL) can also be isolated (137, 138). Consequently, the human tooth can be considered as a treasured supply for MSCs. While all these MSC subpopulations hold great promise for cell-based regenerative applications, the present work, described in this dissertation, puts focus on DPSCs to hold promise for OA treatment and cartilage regeneration.

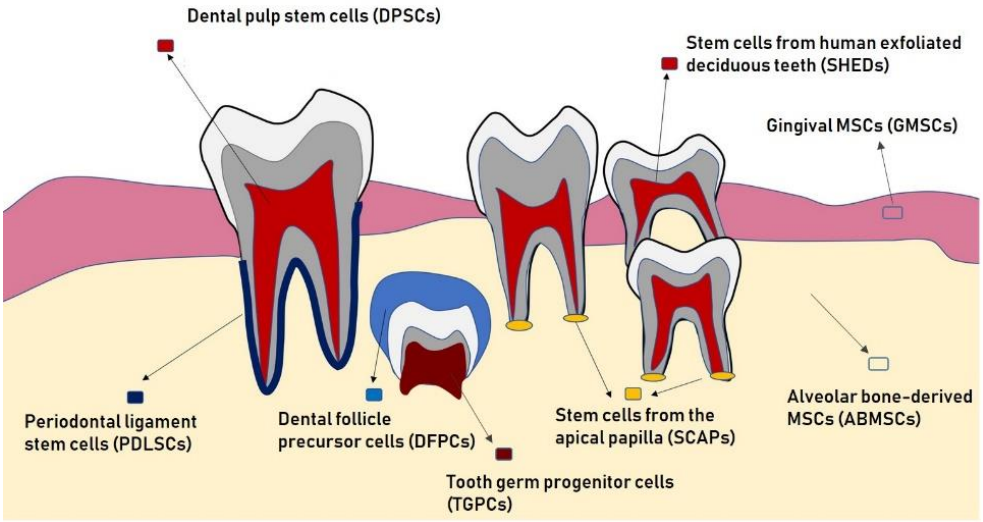

Figure 1.3. Overview of all tooth-associated mesenchymal stem cell (MSC) types. Different sources of MSCs can be distinguished within the human tooth and surrounding tissues; dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs), dental follicle precursor cells (DFPCs) and tooth germ progenitor cells (TGPCs). Additionally, two other MSC subtypes can be found in the gingiva and alveolar bone; gingival MSCs (GMSCs) and alveolar bone-derived MSCs (ABMSCs). In addition, stem cells from the pulp of human exfoliated deciduous teeth (SHEDs) can also be isolated. *This image was adapted from Wang et al. (139).*

Gronthos *et al.* demonstrated the capacity of DPSCs to generate dentin both *in vitro* and *in vivo* (130). Therefore, DPSCs were initially considered for possible applications in regeneration of dental-associated tissues. Following reports revealed their MSC-like characteristics, including their immunophenotyping,

plastic adherence and the ability to differentiate into classical mesodermal cell lineages; adipocytes, osteocytes and chondrocytes *in vitro* (39, 92). In opposite to BM-MSCs, DPSCs showed a higher proliferative rate and have an easy isolation procedure by which they can be harvested (39). In addition, the immunomodulatory properties of DPSCs display their promise as cell-based therapies for immune and inflammation-related diseases (140-143).

DPSCs have been described previously as a promising cell source for hyaline cartilage restoration. Mata and colleagues showed that differentiated DPSCs express collagen type II and aggrecan *in vitro* and, when cultured in alginate hydrogels and implanted in a rabbit model, DPSCs resulted in significant cartilage regeneration (144). Numerous scaffolds have been utilized to emphasize the chondrogenic differentiation capacity of DPSCs, including hydrogels containing poly(ethylene glycol) dimethacrylate (PEGDMA), methacrylated gelatin (GelMA), HA- (145) and chitosan-based scaffolds (146). In addition, Dai *et al.* reported that costal chondrocytes combined with exogenous FGF-9 are suitable to supply chondro-inductive stimuli to DPSCs *in vitro* and *in vivo* (147). Rizk and colleagues showed that TGF-β3-transduced DPSCs express chondrogenic markers *in vitro* and when seeded on poly-l-lactic acid/polyethylene glycol (PLLA/PEG) electrospun fiber scaffolds *in vivo* (148). Chen *et al.* demonstrated the successful chondrogenic differentiation by SHEDs *in vitro* and the ability to generate new cartilage-like tissues after subcutaneous transplantation in nude mice (149). Yu and colleagues showed that the *in vivo* transplantation of rat STRO-1^+ DPSCs at the 1st passage developed into dentin, bone and cartilage structures (150). Also paracrine-mediated effects have been attributed to DPSCs for cartilage regeneration and beneficial effects in arthritis-related diseases. In an experimental mouse model of rheumatoid arthritis (RA), clinical assessment revealed minimal paw swelling after treatment with SHEDs (151). In dogs diagnosed with OA, multiple IA injections of puppy deciduous teeth stem cells (pDSCs) were performed. Their outcomes showed that IA injection considerably reduced pain and lameness, and prevented OA progression (152). In horses, equine dental pulp connective tissue particles showed a remarkable decrease in lameness for at least two weeks. Comfort scores were improved between, before, and 45 days after pulp injection (153).

Despite the fact that substantial reports put light on their chondro-regenerative capacities, data showing paracrine-mediated chondro-salvaging or protective effects of DPSCs are limited. Future investigations elucidating the DPSC-mediated effects by which they might aid in OA should therefore be implemented.

1.4. Platelet Concentrates for Cartilage Regeneration and Osteoarthritis

In articular cartilage, numerous growth factors act in synergy to control development and homeostasis of the tissue throughout life. Therefore, growth factors have been proposed as promising treatments for enhanced regeneration of cartilage or in inflammatory situations such as in OA. Different growth factors including TGF-β, basic (b) FGF, bone morphogenetic proteins (BMPs) and platelet derived growth factor (PDGF) have been described to have favourable effects on hyaline cartilage repair (reviewed in (154)). However, the administration of individual growth factors or cytokines has different disadvantages, including short time of activity, requiring the injection of massive amounts of growth factors, and multiple injections (155, 156). A growing amount of researchers are focusing on natural growth factor reservoirs, such as platelet concentrates. The usage of platelet concentrates is rising in different medical fields, because of their availability, cost-effectiveness, and their autologous nature (157). Moreover, nowadays, research in the tissue engineering field aims on the identification of useful scaffolds to address the requirements of adequate healing of large cartilage defects. Among biomaterials, gel-like platelet derivatives might reach particular attention.

Platelet concentrates were already utilized decades ago; fibrin glue, for instance, was already used as a surgical additive in the 1970's. During the years, several different techniques have been investigated and every technique resulted in a different platelet-derivative varying in growth factors secretion levels, leukocyte content and fibrin matrix. Platelet concentrates can be divided into first and second generation platelet concentrates (158).

1.4.1. First Generation Platelet Concentrates

The first blood derivatives used were the fibrin glues or fibrin sealant. They are composed of concentrated fibrinogen, thrombin, and calcium chloride. Fibrin glues can be used as tissue adhesives for a variety of surgical procedures. Their widespread use has been hampered by their weakness compared to other sealants and the high costs associated to processing autologous blood. These drawbacks added to the development of platelet rich plasma (PRP), an autologous blood derivative, which combines the fibrin properties of fibrin sealants with the presence of platelets. PRP can be used as a liquid solution or as a gel and both forms have a low density fibrin network. Despite the wide variety of clinical applications, there are some disadvantages associated to PRP. The production requires the use anti-coagulants and the use of bovine thrombin. Moreover, there is a variety of available preparation protocols, which result in different end products without proper standardization (158, 159).

1.4.2. Second Generation Platelet Concentrates

Platelet-rich fibrin (PRF) is a second generation platelet concentrate. This group of platelet derivatives is made up by two subtypes: 'pure platelet-rich fibrin (P-PRF)' without leukocytes and 'leukocyte- and platelet-rich fibrin (L-PRF)' with leukocytes. In contrast to PRP, the production of L-PRF does not require any biochemical handling and is generated by one single centrifugation step of whole blood without the supplementation of anti-coagulants. Table 1.1. offers an overview comparing L-PRF, to P-PRF and PRP about their most important properties (159, 160). L-PRF offers a simple and cost effective substitute to PRP. After centrifugation, three different parts can be distinguished: red blood cells at the bottom, an acellular plasma part and the L-PRF clot in the middle of the tube (158, 159). Despite the wide variety of clinical implementations, the present dissertation focusses on the chondrogenic potential of L-PRF (Figure 1.4).

Table 1.1. Overview table comparing L-PRF, P-PRF and PRP about their most important characteristics.

	L-PRF	***P-PRF***	***PRP***
Protocol	Easy	Complex	Complex
Speed-rate	Fast	Fast	Slow
Use of anti-coagulants	No	Yes	Yes
Costs	Low	High	High
Fibrin	High	High	Low
Polymerization	Strong	Strong	Weak
Presence of leukocytes	High	Poor	Moderate
Immunomodulation	High	Poor	Poor
Angiogenic effects	High	High	Moderate
Mechanical properties	Strong	Strong	Moderate

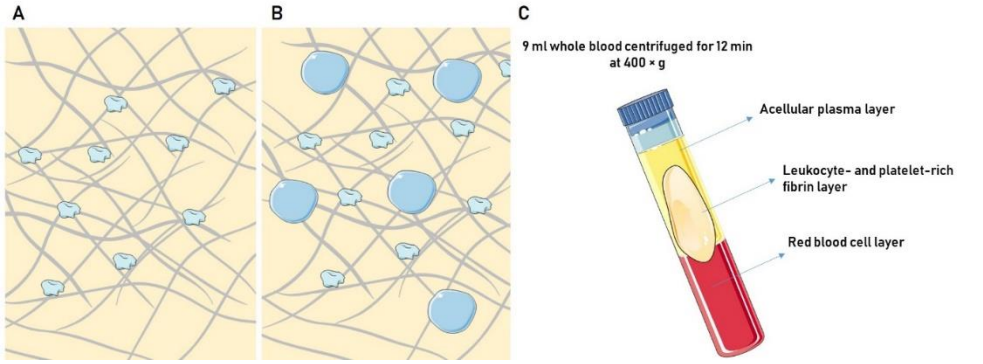

Figure 1.4. Schematic illustration of the pure platelet-rich fibrin (P-PRF) and leukocyte- and platelet-rich fibrin (L-PRF) architecture and the production protocol for L-PRF. (A-B) PRF can exist in two forms: P-PRF (A) and L-PRF (B). They can be distinguished based on the presence of leukocytes. Leukocytes are displayed as the blue larger circles, platelets are the light blue smaller structures and the fibrin network is visualised by the grey-orange thick structures. (C) One-step protocol for the generation of L-PRF. After collection of whole blood, tubes must be immediately centrifuged, which results in three layers; red blood cells, the L-PRF clot in the middle of the tube and an acellular plasma fraction. *This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.*

1.4.3. The Chondrogenic Properties of Leukocyte- and Platelet-Rich Fibrin

L-PRF consists of three components: the leukocytes portion, platelets and the fibrin matrix, all of which can have a beneficial influence on cartilage regeneration or OA. The leukocytes present in L-PRF have an influence not only because of their immune potential but also because these cells are important mediators of the wound healing processes. Leukocytes can secrete various cytokines or growth factors, influencing chondrogenesis or inducing a positive effect on the underlying immunological process of OA. They have been reported to induce an overproduction of some growth factors, including vascular endothelial growth factor (VEGF) and TGF-β1 (161).

Platelets are significant sources of growth factors and other biomolecules which can stimulate cartilage regeneration or induce proliferation and activation of other cells involved in the OA pathophysiology (162). Platelets contain three types of organelles: lysosomes, dense granules and alpha granules. These alpha granules contain platelet-specific proteins, cytokines, growth factors, angiogenic factors and PGs. Upon degranulation, platelets release various growth factors, which have also been reported to enhance tissue healing, cartilage homeostasis and immunomodulation (154, 163). These growth factors comprise; PDGF, IGF-1, TGF-β1, bFGF, epidermal growth factor (EGF) and VEGF, all that have been described to positively influence chondrogenesis and chondrocytes (164).

The third component of L-PRF is the fibrin matrix. This matrix does not only capture the factors released by the platelets and leukocytes, which offers a slow release over time, but it also offers a suitable scaffold during tissue regeneration. Therefore, when it comes to healing of a cartilage defect, the fibrin matrix might be of particular interest.

To date, numerous studies have mainly investigated the cartilage regenerative potential of other platelet derivatives, such as PRP (165). *In vitro* studies demonstrated their positive effects on chondrocyte proliferation and deposition of cartilage matrix (166, 167). Several preclinical animal studies revealed positive effects on cartilage repair induced by PRP (168). *In vitro* studies of the chondrogenic potential of L-PRF are limited (164). Some trophic and protective

effects by PRF on chondrocytes have previously been demonstrated (169-171), and one *in vitro* study showed the chondro-inductive effect of the eluate from fibrin-rich plasma membrane on AT-MSCs (172). L-PRF was also tested in preclinical animal studies for cartilage repair by several previously. The platelet concentrate was tested for repair of chondral, osteochondral and menisci defects in mainly rabbit and dog models. Overall, preclinical studies demonstrated promise for cartilage repair after PRF treatment was combined with autologous cartilage or MSCs (164). However, little is known about the mechanisms of action and biological features of L-PRF on chondrogenesis and on chondrocytes.

1.5. Tendon Injuries

1.5.1. Pathophysiology and Current Treatments

Tendons function in transmitting forces from skeletal muscle to bone as well as providing stability to the joint (173). Tendon injuries are a common clinical matter in both human and veterinary medicine. Tendon development depends on the interplay of growth factors, transcription factors and tension during development. Tendons are primarily made up of collagen with tenocytes residing in between the fibres. 70% of the tendon is water, while 30% is dry mass, of which collagen type I accounts for 65-80%, and elastin for approximately 2%. Tendon is not only made up of type I collagen but, to a lesser extent, also other collagens, such as collagen type III, IV, V, and VI, are present. The ECM is composed of several PGs, glycoproteins, and other smaller molecules (173, 174). Decorin and biglycan are PGs and function in the organisation of collagen fibre bundles. Other tendon-associated PGs include fibromodulin and lumican. Tenascin C is a glycoprotein, which is regulated by mechanical stimulation and is key in collagen fibre alignment and orientation. Another tendon-related glycoprotein is tenomodulin, which is crucial in proliferation and maturation of tendon cells. The expression of tenomodulin is positively regulated by scleraxis. Scleraxis, together with Mohawk, and early growth response protein 1 have been recognised as crucial transcription factors involved in the development of tendon tissue (175).

Current treatment strategies are inadequate in restoring the function the tissue exerted before. They consist out of nonsteroidal anti-inflammatory drug injection, physiotherapy, or surgery (10, 174). Tendon tissue engineering has been

proposed as a promising technique for tendon repair. MSCs, including tendon-derived stem cells (TDSCs), have been widely studied in tendon regenerative fields, because of their prominent differentiation capacity, expression of tendon markers, and remarkable self-renewal ability (176). Although many genes are reported to be involved in tendon development, they are not solely expressed in tendon tissues. Moreover, because of this limited knowledge in tendon-specific markers, transcription factors and signalling pathways, there is a lack of a standardized method for tenogenic differentiation (176).

1.6. *In Vitro* Evidence of Tenogenic Differentiation of Stem Cells

In contrast to chondrogenic, adipogenic, and osteogenic differentiation systems, there is no adequate tenogenic differentiation method. Several growth factors associated with tendon development, namely endothelial growth factor, VEGF, bFGF, PDGF, and TGF-β, have been utilized to drive MSCs towards the tenogenic lineage. In addition to growth factors, mechanically loaded cultures and various scaffolds have been widely employed to study tenogenesis by MSC subtypes (177). Moreover, co-culture systems between MSCs and TDSCs have also been applied (175, 176). However, studies investigating the combined effect of growth factors and 3D culture, and the combination with tension or mechanical stimulation, are paving the way towards increased knowledge of differentiating MSCs into the tenogenic lineage and contributes to the knowledge on the ideal MSC source for tendon-regenerative applications. The most investigated MSC subtypes include BM-MSCs, AT-MSCs and TDSCs.

BM-MSCs are known to express several tendon-associated markers, such as tenascin C and collagen type I (178). Co-culture models with TDSCs demonstrated a crosstalk between both cell types to induce a tenogenic phenotype via the upregulation of tenogenic markers (179, 180). BM-MSCs have been demonstrated to form 3D embryonic tendon-like tissue *in vitro* via the usage of fixed-length fibrin gels and TGF-β3 signalling (181). Furthermore, paracrine-mediated impacts on tendon cells have also been investigated *in vitro* and *in vivo* (182). However, ectopic ossification is one of the major drawbacks of utilizing BM-MSCs for soft-tissue regeneration (183). In attempt to overcome this, AT-MSCs established an

interesting candidate cell source for tendon tissue engineering. They can be isolated from subcutaneous adipose tissue or from liposuction aspirates (184, 185). Comparable to BM-MSCs, co-culture models established a cellular crosstalk leading to an up-regulation of tendon-related markers (186). Tenogenic differentiation of AT-MSCs has been described upon stimulation by different growth factors in monolayer, (dynamic) scaffold cultures, mechanically loaded systems or variations in oxygen tension (187-190). In addition, they have been described to aid in tendon repair via the prevention of ectopic bone formation, inhibition of inflammation and stimulation of vascularisation (191). Nevertheless, AT-MSCs isolation might produce inhomogeneous cell populations.

TDSCs compose up to 3-4% of the total number of cells in tendons and have been reported to be able to differentiate towards tenocytes (176, 178). Several reports highlighted the possible contribution of tendon stem cell populations toward the generation of tendon-like tissues *in vitro* and *in vivo*, but the mechanisms involved are still to be fully understood (192, 193). Nonetheless, several drawbacks can be associated with the usage of TDSCs, such as limited cell numbers, donor-site morbidity and the purity of tendon cell populations is highly arguable (194).

1.6.1. Dental Stem Cells for Tendon Regeneration

MSCs derived from the dental pulp or periodontal ligament propose alternative stem cell sources for applications in tendon tissue engineering (195, 196). They are of particular interest compared to other MSC sources because of their easy isolation, a higher proliferative ability and immunomodulatory nature, as previously mentioned (39, 130, 197, 198). Several research groups demonstrated that periodontal ligament contains endogenous stem/progenitor cells (132, 199, 200), which express tendon-specific markers such as scleraxis, tenomodulin and tenascin C (132, 201, 202). Tenogenic characteristics of PDLSCs *in vitro* upon growth factor stimulation, including FGF-2, TGF-β1 and BMPs were investigated previously. *In vitro* data indicated that both FGF-2 and growth/differentiation factor (GDF)-5 predominantly differentiate PDLSCs into teno/ligamentogenic lineages (196, 203, 204). Also, DPSCs are shown to express tendon-related markers such as scleraxis, tenascin C, tenomodulin, eye absent homologue (EYA) 2, collagen type I and type VI under static mechanical loading and might be a potential cell source for tendon tissue engineering (195). Nevertheless,

investigating the tenogenic characteristics of PDLSCs in a 3D environment, and comparing them to DPSCs has never been considered so far.

1.7. Aim of the Study

Degenerative diseases and overloading of the joint may harm articular cartilage, eventually leading to OA, and can damage tendon tissue. These tissues are characterized by a limited intrinsic healing capacity and current treatment strategies do not restore full function of the tissue. As the number of patients suffering from OA and tendinopathies are cumulating, there is an increasing need for the development of new treatment strategies for articular cartilage defects, OA, and tendon lesions.

Given the encouraging results of MSCs and platelet derivatives in (pre)clinical research and experimental evidence supporting their therapeutic potential in OA and cartilage repair, a first part of the study focused on gaining more insights on MSC- and platelet concentrate-based therapies for cartilage repair and OA. In contrast to BM-MSCs, DPSCs are a less studied MSC subtype in the field of cartilage regeneration and OA management. Nevertheless, they own superior properties to BM-MSCs and can be obtained with minimal invasive surgical procedures and donor site morbidity. Moreover, *in vitro* studies of the chondrogenic potential of L-PRF are limited, as most research focused on PRP within the field of cartilage regeneration. Because of the chondrogenic differentiation potential and immunomodulatory properties of DPSCs, and the growth factor-rich content of L-PRF, **we hypothesize that DPSCs and L-PRF can both enhance cartilage regeneration *in vitro* and have immunomodulatory effects (Figure 1.5). In Chapter 2** of the current dissertation, we show the *in vitro* chondrogenic differentiation potential of human DPSCs compared to human BM-MSCs. Moreover, we evaluated whether L-PRF had an additive effect on chondrogenesis of both MSC types in a 3D cell-culture system. Secondly, we also assessed the effect of growth factor release of DPSCs and L-PRF on healthy chondrocytes and TNF-α- and IL-1β-stimulated chondrocytes *in vitro*, on viability, OA-related gene expression, cartilage-specific ECM deposition, and inflammatory cytokine secretion.

In a second part of the current dissertation, we focused on assessing the tendon-regenerative capacities of two tooth-associated stem cell types. **In Chapter 3, we evaluate the tenogenic differentiation capacity of DPSCs and PDLSCs and compare them to BM-MSCs**. We **hypothesized that PDLSCs provide a more unique and favourable MSC source over DPSCs and BM-MSCs to synthetize tendon-like constructs *in vitro.*** 3D growth conditions under static tension and the exogenous supplementation of TGF-β3 are investigated to generate tendon-like structures *in vitro*. Cell alignment, cell density, gel contraction and the presence of tendon-related markers were assessed (Figure 1.5).

Before DPSC or L-PRF treatment approaches might be implemented into the clinic, preclinical studies requiring translational large animal models are needed. **In Chapter 4, we discuss the advantages and disadvantages of small and large animal models for translational cartilage repair studies. We also focused on suitable outcome measures for evaluating cartilage repair in preclinical studies**.

Preclinical studies in orthopaedic research for cartilage repair and in OA using the sheep as a large animal model are emerging. This is because of marked similarities of the sheep with human cartilage repair processes and joint organisation. Critical-size defect and OA models have been described in the sheep, and data on ovine MSCs is currently also increasing. However, characterization of ovine MSCs is not fully known and caused some controversy. **In Chapter 5, we aim to isolate ovine MSCs from the dental pulp and assess their MSC-like characteristics**.

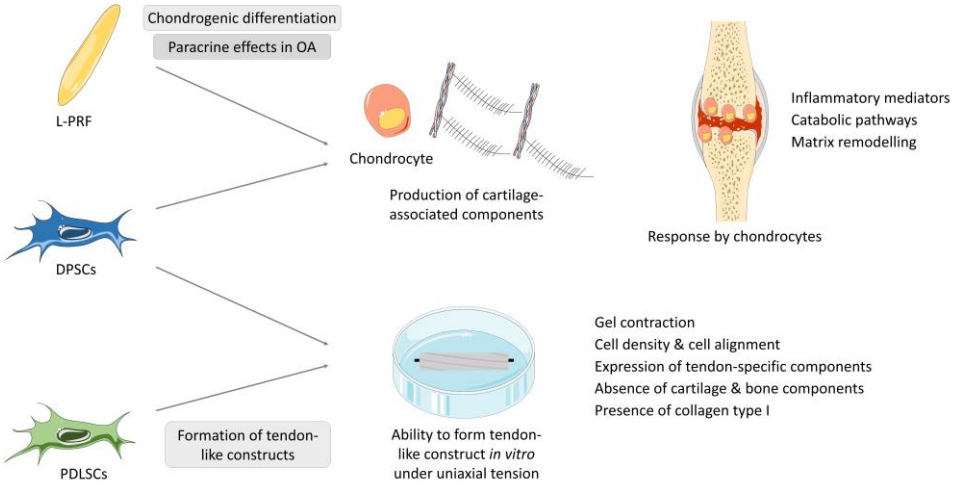

Figure 1.5. Aim of the current dissertation. Osteoarthritis (OA) is a degenerative and inflammatory condition of synovial joints with irreversible loss of supportive cartilage matrix. Dental pulp stem cells (DPSCs) can be differentiated into cartilage-producing cells and secrete numerous growth factors associated with tissue repair and immunomodulation. Moreover, leukocyte- and platelet-rich fibrin (L-PRF), a blood-derived and clinically applied biomaterial, has recently emerged as a promising treatment in regenerative medicine due to its growth factor content and supportive fibrin matrix. Therefore, we hypothesize that DPSCs and L-PRF can both enhance (endogenous) cartilage regeneration *in vitro* and have paracrine-mediated effects. Other common MSDs are tendon-related pathologies. Spontaneous tendon healing results in the formation of a scar-like tissue with inferior structural and mechanical properties. In a second part of the current study, we focus on the tenogenic differentiation capacity of DPSCs and periodontal ligament stem cells (PDLSCs) and compare them to bone marrow-derived mesenchymal stem cells (BM-MSCs). Three-dimensional (3D) growth conditions under static tension and the exogenous supplementation of transforming growth factor-beta 3 (TGF-β3) are investigated to synthetize tendon-like structures *in vitro*. *This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.*

Chapter 2: Therapeutic Potential of Dental Pulp Stem Cells and Leukocyte- and Platelet-Rich Fibrin for Osteoarthritis

Based on;

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2.1. Abstract

Osteoarthritis (OA) is a degenerative and inflammatory joint disorder with cartilage loss. Dental pulp stem cells (DPSCs) can undergo chondrogenic differentiation and secrete growth factors associated with tissue repair and immunomodulation. Leukocyte- and platelet-rich fibrin (L-PRF) emerges in regenerative medicine because of its growth factor content and fibrin matrix. This study evaluates the therapeutic application of DPSCs and L-PRF in OA via immunomodulation and cartilage regeneration. Chondrogenic differentiation of DPSCs, with or without L-PRF exudate (ex) and conditioned medium (CM), and of bone marrow-derived mesenchymal stem cells (BM-MSCs) was compared. These cells showed differential chondrogenesis. L-PRF was unable to increase cartilage-associated components. Immature murine articular chondrocytes (iMACs) were cultured with L-PRF ex, L-PRF CM, or DPSC CM. L-PRF CM had pro-survival and proliferative effects on unstimulated and cytokine-stimulated iMACs. L-PRF CM stimulated the release of interleukin (IL)-6 and prostaglandin E2 (PGE2), and increased *matrix metalloproteinase* (*MMP)-13*, *tissue inhibitor of metalloproteinase* (*TIMP)-1* and *IL-6* mRNA levels in cytokine-stimulated iMACs. DPSC CM increased the survival and proliferation of unstimulated iMACs. In cytokine-stimulated iMACs, DPSC CM increased *TIMP-1* gene expression, whereas it inhibited nitrite release in three-dimensional (3D) culture. We showed promising effects of DPSCs in an *in vitro* OA model, as they undergo chondrogenesis *in vitro*, stimulate the survival of chondrocytes and have immunomodulatory effects.

2.2. Introduction

Articular cartilage plays key roles in the function of diarthrodial (synovial) joints (4, 6). Cartilage injuries are very common, predominantly in young and active athletes, and particularly in the knee joint (205-207). They are often considered as risk factors for the development of osteoarthritis (OA) in later life, a degenerative and inflammatory condition of the synovial joint with irreversible cartilage loss (6). OA results in disability, particularly in elderly people, and is associated with a large socio-economic burden (208, 209). OA is more prevalent in the female population and increases with age (209). In people over 60 years of age, it is estimated that 9.6% of men and 18% of women have symptomatic OA (210). Unfortunately, long-lasting regeneration of damaged articular cartilage remains an unmet clinical need. Current treatment strategies aim to relieve pain and clinical signs associated with inflammation. However, patients show no long-term improvements (23). With the aim to restore the damaged cartilage tissue, matrix-induced autologous chondrocyte implantation (MACI), a Food and Drug Administration (FDA)-approved technique, has been developed (211). However, there are several disadvantages such as iatrogenic damage and high costs (25, 212, 213). To overcome these problems, the use of innovative autologous biological tissue engineering techniques using stem cells forms an area of large interest in an attempt to achieve articular cartilage regeneration.

Previous preclinical studies focused on the use of induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) to repair articular cartilage, demonstrating beneficial effects mediated via different mechanisms (as previously reviewed by our group (6)). However, because of the ethical implications regarding the usage of iPSCs and the invasive nature of bone marrow-derived mesenchymal stem cell (BM-MSC) isolation, an alternative cell source is of particular interest: dental pulp stem cells (DPSCs) originating from the neural crest-derived mesenchyme residing in the dental pulp (214, 215). Since they are isolated from extracted human third molars, DPSCs can be obtained with minimal donor site morbidity and iatrogenic damage. DPSCs have been classified as MSCs based upon the International Society for Cellular Therapy (ISCT) criteria (216). Apart from the opportunity of DPSCs to provide a cell replacement treatment, they show therapeutic potential in OA through paracrine and trophic influences on

endogenous cells. Current evidence indicates that DPSCs can be differentiated into cartilage-producing cells (39) and secrete numerous growth factors associated with tissue repair and immunomodulation, including vascular endothelial growth factor (VEGF), monocyte chemoattractant protein (MCP)-1, transforming growth factor-beta (TGF-β) and tissue inhibitors of metalloproteinase (TIMPs) (217-219). In addition, their immunomodulatory capacity makes them strong contenders to be used in inflammatory disorders (143), such as OA. Interestingly, intra-articular (IA) injection of DPSCs resulted in anti-inflammatory effects in rheumatoid arthritis (RA) (151). Co-culture of costal chondrocytes and DPSCs combined with fibroblast growth factors (FGF)-9 showed enhanced chondrogenesis and reduced ossification in tissue-engineered cartilage (147). However, until now, no chondrocyte-salvaging or -stimulating properties have been attributed to DPSCs.

In addition, different growth factors including TGF-β, basic (b) FGF, VEGF, bone morphogenetic proteins (BMPs) and platelet derived growth factor (PDGF) have been described to have a beneficial effect on hyaline cartilage repair (154). Platelets are a natural reservoir of such growth factors within the human body (220). Platelet concentrates such as platelet-rich plasma (PRP) and leukocyte- and platelet-rich fibrin (L-PRF), are known to produce a plethora of autologous growth factors and cytokines (159). In recent years, first generation platelet-rich biomaterials such as autologous PRP have been widely studied in order to realise articular cartilage repair (168). *In vitro* studies demonstrated their positive effects on chondrocyte proliferation and deposition of cartilage matrix (166, 167). Several preclinical animal studies revealed positive effects on cartilage repair induced by PRP (168). In contrast to PRP, L-PRF is a second generation platelet concentrate which can be produced rapidly by the collection of autologous blood after one single centrifugation step and without anti-coagulants (158, 159). The generated product is a fibrin clot consisting of three components; leukocytes, platelets and a supportive fibrin matrix (221). Leukocytes and platelets progressively release a high concentration of cytokines and growth factors respectively over time (221, 222). L-PRF might be applied in cartilage engineering studies because of its supportive fibrin matrix, while the leukocytes present in L-PRF might be important in immunomodulatory mechanisms via cytokine secretion. To date, numerous studies have mainly investigated the cartilage regenerative potential of other platelet derivatives, such as PRP (165). *In vitro* studies of the chondrogenic

potential of L-PRF are limited (164). Some trophic and protective effects by PRF on chondrocytes have previously been demonstrated (169-171), and one *in vitro* study showed the chondro-inductive effect of the eluate from fibrin-rich plasma membrane on a stem cell population (172).

Because of the chondrogenic differentiation potential and immunomodulatory properties of DPSCs, and the growth factor-rich content of L-PRF, we hypothesise that DPSCs and L-PRF can both enhance cartilage regeneration *in vitro* and have immunomodulatory effects. In the current study, first, we investigated the *in vitro* chondrogenic differentiation potential of human DPSCs compared to human BM-MSCs. Second, we evaluated whether L-PRF had an additive effect on chondrogenic differentiation of both MSC types in a three-dimensional (3D) cell-culture system. Third, we assessed the effect of growth factor release of DPSCs and L-PRF on healthy chondrocytes and tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β)-stimulated chondrocytes *in vitro*, on viability, OA-related gene expression, cartilage-specific extracellular matrix (ECM) deposition and inflammatory cytokine secretion.

2.3. Materials and Methods

2.3.1. Human Stem Cell Isolation and Culture

Human third molars were obtained with written informed consent from patients (n = 16) of both genders (15-20 years of age) undergoing an extraction procedure for orthodontic reasons at Ziekenhuis Oost-Limburg (ZOL, Genk, Belgium). Written informed consent of minor patients was acquired via their custodians. The study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the medical ethical committee of Hasselt University (Belgium, protocol 13/0104U, date of approval 3 February 2014). The pulp tissue was obtained by means of forceps after mechanically fracturing the teeth. Next, the pulp tissues were minced into small pieces (1-2 mm^3) and DPSCs were isolated via the explant method (39). Cells were maintained in minimal essential medium, alpha modification (αMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/mL Penicillin and 100 µg/mL Streptomycin (Sigma-Aldrich), 2 mM l-glutamine (Sigma-Aldrich) containing 10% heat-inactivated foetal bovine serum (FBS) (Biowest, Nuaillé, France).

BM-MSCs of three different donors (both male and female), between 6 and 12 years old, were kindly provided by Prof. Dr. Cathérine Verfaillie (Stem Cell Institute, KU Leuven, Leuven, Belgium) (isolated from bone fragments (femur)). BM-MSCs were kept in high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 100 U/mL Penicillin and 100 µg/mL Streptomycin containing 10% heat-inactivated FBS.

All stem cells were routinely screened in our lab for the expression of the following markers: CD34 (negative), CD44, CD45 (negative), CD90, CD105 and Stro-1 (negative). Moreover, their trilineage differentiation capacity was evaluated as previously demonstrated by our group (39). All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2-3 days and all cultures were regularly monitored with an inverted phase-contrast microscope Nikon Eclipse TS100 (Nikon Co., Shinjuku, Tokyo, Japan) equipped with a Jenoptik ProgRes C3 camera (Jenoptik, Jena, Germany) with corresponding ProgRes Capture Pro 2.7 software. When reaching 80-90% confluence, cells were harvested using 0.05% trypsin/EDTA (Sigma-Aldrich) and

sub-cultured for further experiments. All experiments were conducted with DPSCs between passages 2 and 8.

2.3.2. Isolation and Culture of Immature Murine Articular Chondrocytes

Immature murine articular chondrocytes (iMACs) were isolated based upon a previously published protocol by Gosset *et al.* (223) and according to the animal welfare guidelines of the ethical committee of Hasselt University (ID 201762K, date of approval 11 November 2017). In short, after euthanasia of 5-6-day-old C57BL/6 wild type mice (*n* = 219), femoral heads, femoral condyles and tibial plateaus were isolated from the hind limbs and placed in phosphate buffered saline (PBS, Lonza, Basel, Switzerland). Isolated cartilage pieces were then incubated twice in 3 mg/mL collagenase D (Sigma-Aldrich) in low glucose DMEM (Sigma-Aldrich) supplemented with 50 U/mL Penicillin, 50 µg/mL Streptomycin and 2 mM l-glutamine (iMAC standard culture medium) for 45 minutes at 37 °C in 5% CO_2. Cartilage pieces were then incubated 0.5 mg/mL collagenase D in iMAC standard culture medium overnight at 37 °C in 5% CO_2. Afterwards, cartilage fragments were passed through 25 mL, 10 mL, 5 mL and 2 mL pipettes to disperse any cell aggregates. After passing through a 70-µm cell strainer, the cells were centrifuged at 400 × g for 10 min. Cells were resuspended in iMAC standard culture medium supplemented with 10 % heat-inactivated FBS.

Phenotypic characterization was performed by means of immunocytochemistry (ICC) and histological staining. In short, 26.32 × 10^3 cells/cm^2 were seeded on glass or plastic (Thermanox®; Electron Microscopy Sciences, Hatfield, PA, USA) cover slips for 96 h in iMAC standard culture medium supplemented with 10% FBS. Afterwards, they were fixed using 4% paraformaldehyde (PFA) for 20 minutes for ICC or using 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) at 4 °C for transmission electron microscopy (TEM) processing. Immune-reactivity for collagen type II was demonstrated by ICC. Culture purity was assessed by determining the fraction of collagen type 2-positive cells using ImageJ software (The National Institute of Health, MD, USA). The presence of proteoglycans (PGs) was demonstrated via alcian blue, toluidine blue and safranin O staining. All experiments were performed with freshly isolated iMACs.

2.3.3. L-PRF Isolation

Blood samples were obtained from 11 healthy donors from both genders (aged 23-37) (n = 11) with written informed consent. The study protocol and consent procedure were approved by the medical ethical committee from Hasselt University and the Clinical Trial Centre from KU Leuven (S58789/B322201628215, date of approval 21 March 2016). All experiments were performed in accordance with relevant guidelines and regulations. Blood samples were drawn by venipuncture and collected in glass-coated plastic tubes (VACUETTE 9 ml Z Serum Clot Activator Tubes, Greiner Bio-One, Vilvoorde, Belgium). Samples were immediately centrifuged for 12 minutes at 2700 rpm (400 × g) (IntraSpin™ Centrifuge, Intra-Lock, Boca Raton, FL, USA) (Tubes were put per two into the centrifuge directly after drawing blood). The L-PRF clots were removed from the tubes using sterile forceps and separated from the red blood cell phase with an iris spatula (Fine Science Tools, Heidelberg, Germany) (Figure 2.1).

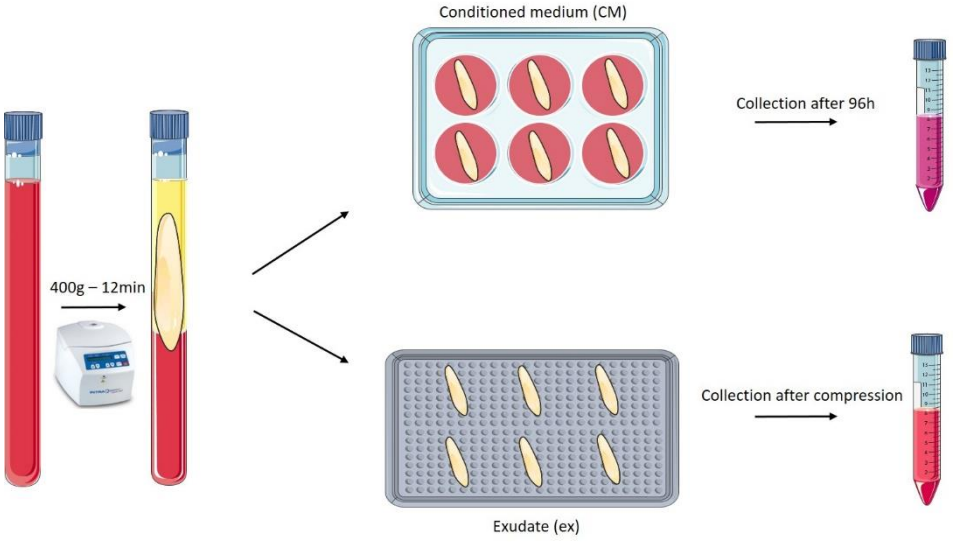

Figure 2.1. Single step production protocol for leukocyte- and platelet-rich fibrin (L-PRF) and the generation of L-PRF conditioned medium (CM) and L-PRF exudate (ex). After collection, whole blood is immediately centrifuged (400 × g) for 12 min, resulting in three different compartments within the tube: red blood cells at the base, the L-PRF clot in the middle of the tube and an acellular plasma portion on top. For the generation of L-PRF CM, L-PRF clots are placed in medium for 96 h. Afterwards, the medium is collected, centrifuged and stored until further use. For L-PRF ex collection, the L-PRF clots are put to

a sterile box and compressed, thereby releasing the exudate, which is collected and stored until further usage. *This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.*

2.3.4. L-PRF Conditioned Medium and Exudate

For the production of L-PRF conditioned medium (L-PRF CM), L-PRF clots were placed in 6 ml of serum-free low glucose DMEM or DMEM/F12 (Thermo Fisher Scientific, Erembodegem, Belgium) supplemented with 2 mM L-glutamine, 50 or 100 U/mL Penicillin and 50 or 100 µg/mL Streptomycin. After 96 h, the medium was collected, centrifuged for 6 minutes at 300 × g, sterile filtered (0.2 µm, Sarstedt, Nümbrecht, Germany) and stored at -80 °C until further use. For L-PRF exudate (L-PRF ex) collection, the L-PRF clots were brought to a sterile box (Xpression™ Fabrication Box, Intra-Lock) and compressed. The weighted press of the box converted the L-PRF clot into a membrane and the exudate was released from the clot, which was collected, sterile filtered and stored at -80 °C until further usage (Figure 2.1).

2.3.5. Chondrogenic Differentiation

Chondrogenic differentiation of DPSCs and BM-MSCs was induced according to the manufacturer's instructions (StemXVivo Human/Mouse Chondrogenic Supplement, R&D systems, BioTechne, Minneapolis, MN, USA). A pellet containing 2.5×10^5 cells in a 15 mL conical tube was subjected to chondrogenic differentiation medium consisting of DMEM/F12 supplemented with 1% insulin transferrin selenite (R&D systems) and 1% chondrogenic supplement (R&D systems). This supplement consists of dexamethasone, ascorbate-phosphate, proline, pyruvate and TGF-β3 with concentrations determined and validated by the manufacturer. To determine the effect of L-PRF on the chondrogenic differentiation, L-PRF ex (3%) and L-PRF CM (5% and 25%) were added to the differentiation medium. Positive and negative controls contained standard differentiation medium with or without the chondrogenic supplement respectively. Every 2-3 days, the medium was changed. The cells were allowed to differentiate for 21, 28 or 35 days, after which the pellets were either fixed with 4% PFA for immunohistochemical (IHC) analysis or with 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) at 4 °C for TEM processing. Percentage alcian blue and

aggrecan stained area was quantified using Image J (The National Institute of Health, MD, USA).

2.3.6. DPSC Conditioned Medium

Conditioned medium of DPSCs (DPSC CM) was prepared by seeding human DPSCs at a density of 20 × 10^3 cells/cm² in iMAC standard culture medium supplemented with 10% FBS. Cells were allowed to attach overnight. Afterwards, cells were rinsed twice with PBS and 1 mL/5 cm² iMAC serum-free standard culturing medium was added. 48 h later, the medium was collected, centrifuged at 161 × g for 6 minutes and stored at -80 °C.

2.3.7. Cell Survival and Proliferation Assay

iMACs were seeded in triplicate in flat bottom 96 well plates at a density of 19.69 × 10^3 cells/cm² or 29.41 × 10^3 cells/cm² for survival and proliferation assays respectively and were allowed to attach overnight. Hereafter, cells were washed twice with PBS and culture medium supplemented with L-PRF ex (1%, 3%, 5%), L-PRF CM (5%, 25%, 50%), or DPSC CM was added. For survival assays, the cells were cultured in serum-free conditions. For proliferation assays, experimental conditions were supplemented with 2% FBS. Negative and positive controls consisted of iMACs cultured in serum-deprived medium (0% or 2% for survival and proliferation respectively) or medium supplemented with 10% FBS respectively.

For cytokine-stimulated iMACs, cells were seeded at a density of 29.41 × 10^3 cells/cm^2 and were allowed to adhere overnight. Subsequently, cells were washed twice with PBS and stimulated with the inflammatory cytokines recombinant mouse TNF-α (10 ng/mL) and recombinant mouse IL-1β (10 ng/mL) (Immunotools, Friesoythe, Germany) for 24 h. Hereafter, experimental conditions were added, including inflammatory cytokines TNF-α and IL-1β (10 ng/mL). Unstimulated conditions received no cytokines.

The effect of L-PRF ex, L-PRF CM and DPSC CM on iMAC viability was evaluated using propidium iodide (PI, Sigma-Aldrich). After 24, 48, or 72 h, cells were lysed using Reagent A100 (Chemometec, Lillerød, Denmark). Next, cells were incubated with PI (diluted 1/50 in Reagent B (Chemometec)) for 15 minutes in the dark.

Solutions were transferred to a black 96-well plate with clear bottom (Greiner bio-one) and fluorescence intensity was measured at an excitation wavelength of 540 nm and an emission wavelength of 612 nm (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany).

2.3.8. Reverse Transcriptase Quantitative Polymerase Chain Reaction

iMACs were seeded at a cell density of 52.63 × 10^3 cells/cm^2 and left to adhere overnight. The cells were subsequently washed twice with PBS and stimulated with inflammatory cytokines recombinant mouse TNF-α (10 ng/mL) and recombinant mouse IL-1β (10 ng/mL) for 24 h. Afterwards, experimental conditions were added containing TNF-α and IL-1β (10 ng/mL). Unstimulated conditions received no cytokines. All cells were cultured in 2% FBS.

After 24 h, medium was collected, centrifuged and stored at −80 °C for enzyme-linked immunosorbent assay (ELISA) experiments, while RNA was extracted from total cell lysates using the RNeasy Mini Kit (74104, Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. After reverse transcription to cDNA using qScript cDNA Supermix (Quanta Bioscience, Carlsbad, CA, USA), a quantitative PCR was conducted on a StepOnePlus detection system (Applied Biosystems, Foster City, CA, USA) using standardised cycling conditions (20 s at 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C). Primer sequences are listed in Table 2.1.

Table 2.1. The primers used for reverse transcriptase quantitative polymerase chain reaction analysis.

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'	Accession number
ADAM-17	AGAGAGCCATCTGAAGAGTTTGT	CTTCTCCACGGCCCATGTAT	NM_009615.6
ACAN	GTCGCTCCCCAACTATCCAG	AAAGTCCAGGGTGTAGCGTG	NM_001361500.1
COL IIa1	GAAGGATGGCTGCACGAAAC	AATAATGGGAAGGCGGGAGG	XM_006520386.3

IL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC	NM_031168.2
iNOS	CCCTTCAATGGTTGGTACATGG	ACATTGATCTCCGTGACAGCC	NM_001313922.1
MMP-13	TCGCCCTTTTGAGACCACTC	AGCACCAAGTGTTACTCGCT	NM_008607.2
TGF-β	GGGCTACCATGCCAACTTCTG	GAGGGCAAGGACCTTGCTGTA	NM_011577.2
TIMP-1	TCCTAGAGACACACCAGAGCA	AGCAACAAGAGGATGCCAGA	NM_001294280.2
TNF-α	GTCCCCAAAGGGATGAGAAGT	TTTGCTACGACGTGGGCTAC	NM_013693.3
Housekeeping gene	**Forward primer 5'-3'**	**Reverse primer 5'-3'**	**Accession number**
CYPA	GCGTCTCCTTCGAGCTGTT	AAGTCACCACCCTGGCA	NM_008907.2
HMBS	GATGGGCAACTGTACCTGACTG	CTGGGCTCCTCTTGGAATG	NM_001110251.1
HPRT	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC	NM_013556.2

2.3.9. Enzyme-Linked Immunosorbent Assay

ELISAs were performed for IL-6 and prostaglandin E2 (PGE2) (R&D systems). ELISAs were performed according to the guidelines of the manufacturer. The absorbance of the end product was measured with a plate reader (FLUOstar OPTIMA and iMARK Microplate Reader, Biorad, Temse, Belgium). To ensure that the measured concentrations in L-PRF ex, L-PRF CM and DPSC CM were iMAC-derived, the conditions were included in the ELISA experiment as a control.

2.3.10. Nitrite Measurements

iMACs were seeded at a density of 52.63 × 10^3 cells/cm^2 and were allowed to adhere for 24 h. Cells were subsequently washed twice met PBS and stimulated with the inflammatory cytokines TNF-α (10 ng/mL) and IL-1β (10 ng/mL). After 24 h, experimental conditions were added containing TNF-α and IL-1β (10 ng/mL). Unstimulated conditions received no cytokines. All cells were cultured in 2% FBS. After another 24 h, the medium was collected, centrifuged and stored at −80 °C.

Nitrite was quantified using the Griess Reagent System (Promega Benelux B.V., Leiden, The Netherlands) according to the manufacturer's guidelines. Absorbance was measured at a wavelength of 540 nm using a plate reader (FLUOstar OPTIMA).

2.3.11. Three-Dimensional Culture of iMACs

For cytokine-stimulated iMAC pellets, 5×10^5 iMACs were washed twice and resuspended in culture medium containing recombinant mouse TNF-α (10 ng/mL) and recombinant mouse IL-1β (10 ng/mL). Cells were centrifuged in 15 ml polypropylene tubes at 400 × g and maintained at 37 °C under 5% CO_2. The caps of the tubes were loosened to allow for air exchange. 24 h later, the medium was replaced for the experimental conditions with TNF-α and IL-1β (10 ng/mL). Unstimulated conditions received no cytokines. All conditions were cultured in 2% FBS. 72 h later, the medium was collected, centrifuged and stored at −80 °C for nitrite measurements, while pellets were fixed with 4% PFA for IHC.

2.3.12. Transwell Migration Assay

iMACs were seeded in 24 well plates in iMAC standard culturing medium at a density of 26.32 x 10^3 cells/cm^2, and allowed to attach overnight. The day after, cells were washed with PBS and the medium was changed to serum-free standard culture medium. Positive and negative controls consisted of iMAC standard culturing medium supplemented with 10% or 0% FBS respectively. 24 h later, inserts (ThinCert™, 8 µm pore size, Greiner Bio-One) were coated with 0.01 mg/ml poly-L-lysin (Sigma-Aldrich) for 1.5 h and washed with MilliQ and PBS. 52.63 x 10^3 cells/cm^2 DPSCs, suspended in iMAC standard culturing medium supplemented with 0% FBS, were seeded in the inserts. After 24 h, the transmigrated cells were fixed with 4% PFA and stained with 0.1% crystal violet. Migration was quantified with AxioVision software (Carl Zeiss, Aalen, Germany).

2.3.13. Immunocytochemical Staining

For collagen type II expression in iMACs, permeabilisation and blocking occurred simultaneously with 10% protein block (DAKO, Glostrup, Denmark) and 0.2% Triton in PBS for one hour. Cells were then incubated with a rabbit anti-collagen type II antibody (ab34712, polyclonal, 1:100, Abcam, Cambridge, UK) diluted in

10% protein block in PBS for one hour at room temperature (RT). Negative controls were included that were omitted of primary antibody. Afterwards, they were incubated with the Alexa 555-labelled donkey anti-rabbit IgG (A31572, Thermo Fisher Scientific) diluted 1/500 in PBS for 30 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) for 10 min. Samples were mounted using fluorescence mounting medium (DAKO). Pictures were taken with a Leica DM4000 B Microscope (Leica Microsystems, Wetzlar, Germany).

2.3.14. (Immuno)histology

Immunohistochemistry

Cartilage pellets were embedded in paraffin and 7 µm thick sections were cut. Samples were deparaffinised in xylene and ethanol baths (xylene: 2 times 5 min, ethanol: 100%, 100%, 95%, 80%, 70%, 50%, 2 minutes each). Antigen retrieval was performed by heating the samples three times for 5 minutes in 1 × target retrieval solution (DAKO). In case of 3,3'-Diaminobenzidine (DAB, DAKO) staining, peroxidase block (DAKO) was used for 20 minutes. Next, nonspecific binding of the antibodies was inhibited with protein block (DAKO) for 30 minutes at RT. Samples were then incubated with a rabbit anti-aggrecan antibody (ab186414, clone number EPR14664, 1:500, Abcam) diluted in 10% protein block in PBS for one hour at RT. Subsequently, samples were incubated with the advance HRP Link System (K4067, DAKO) for 30 minutes at RT. Hereafter, samples were incubated with DAB for 5 minutes and counterstained with haematoxylin for 8 minutes after which they were washed with running tap water for 20 min.

Histology

For histological analyses routinely used safranin O, alcian blue, toluidine blue and Masson's trichrome staining were performed.

Safranin O

Samples were incubated with haematoxylin for 8 minutes, and washed with running tap water for 20 minutes. Next, they were incubated with 0.05% Fast Green solution for 5 minutes and rinsed with 1% acetic acid solution for 10

seconds. Thereafter, the samples were incubated in 0.1% Safranin O solution (Merck, Overijse, Belgium) for 5 minutes.

Alcian blue

Samples were incubated with Alcian blue solution (pH = 2.5) for 30 minutes at room temperature (RT). Subsequently, samples were washed with running tap water for 10 minutes and submerged in distilled water for 1 minute. Next, nuclear fast red solution was applied for 10 minutes and samples were dipped for 1 second in distilled water.

Toluidine blue

Samples were incubated with 1% Toluidine blue solution for 20 minutes at RT. Subsequently, samples were washed with distilled water for 30 seconds.

Masson's trichrome

After incubation with haematoxylin and running tap water, samples were incubated in Ponceau/Fuchsine solution for 5 minutes. Next, samples were incubated in 1% phosphomolybdic acid and Aniline blue solution for 5 minutes each. After incubation in 1% phosphomolybdic acid for 5 minutes, samples were placed in acetic acid for 2 minutes. Between each incubation, samples were washed with distilled water.

All samples were dehydrated in ethanol and xylene (ethanol: 70%, 80%, 95%, 100%, 100%, 100% xylene, 100% xylene, 2 minutes each) and mounted using DPX (Merck, Darmstadt, Germany). Slides were visualised with the Mirax slide scanner (Carl Zeiss NV-SA, Zaventem, Belgium) using the Mirax scan software. Photos of scanned slides were made with the Mirax viewer (Carl Zeiss NV-SA) or images were taken with a Leica DM2000 LED Microscope.

2.3.15. Transmission Electron Microscopy

Samples were processed for TEM imaging as described previously (224). After fixation, the fixative was aspirated with a glass pipette, and samples were postfixed in 2% osmium tetroxide for one hour. Subsequently, samples were placed through a dehydrating series of graded concentrations of acetone. Dehydrated samples were impregnated overnight in a 1:1 mixture of acetone and

araldite epoxy resin at RT. After impregnation, samples were embedded in araldite epoxy resin at 60 °C and monolayer samples were embedded in araldite according to the popoff method (225). Ultrathin sections (0.06 µm) were mounted on 0.7% formvar-coated copper grids (Aurion, Wageningen, the Netherlands), contrasted with 0.5% uranyl acetate and a stabilised solution of lead citrate using a Leica EM AC20 (Leica). Samples were observed using a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Netherlands) equipped with a Morada Soft Imaging System camera with corresponding iTEM-FEI software (Olympus SIS, Münster, Germany).

2.3.16. Statistical Analysis

Statistical analysis was performed using Graphpad Prism 7.04 software (Graphpad, San Diego, CA, USA). Normality was tested using the Shapiro-Wilk and the D'Agostino and Pearson normality test. Normal distributed data were tested with one-way analysis of variance (ANOVA) or two-way ANOVA and Dunnet's multiple comparison post-test. Nonparametric data were analysed with the Kruskal-Wallis test followed by Dunn's post-test. "n" represents the number of experiments (for every experiment a different DPSC/L-PRF donor was used). Any p-value ≤ 0.05 was considered to be statistically significant. All data were presented as mean ± standard error of mean (S.E.M.).

2.4. Results

2.4.1. Differences in Chondrogenic Differentiation Potential Between BM-MSCs and DPSCs and the Effect of Exposure to L-PRF During Chondrogenesis

In order to compare the chondrogenic differentiation potential between human DPSCs and BM-MSCs, cells were subjected to a 3D chondrogenic differentiation system over 21 days. To test the effect of exposure to L-PRF during chondrogenic differentiation, cells were subjected to the same 3D differentiation system, but supplemented with L-PRF ex (3%) or L-PRF CM (5% and 25%) for 21 days (n = 3). Following the three week culture, both cell types formed compact 3D micromasses under all experimental conditions (Figure 2.2A). IHC revealed abundantly present ECM surrounding both differentiated stem cell types (Figure 2.2A). Ultrastructural analyses of the 3D micropellets of both cell types showed the presence of dense matrix-filled vesicles, suggesting glycosaminoglycan (GAG) production (Figure 2.2B, arrowheads). This was supported by the alcian blue staining which demonstrated the presence of GAGs in the ECM of both differentiated MSC types (Figure 2.2C). Quantitative analysis of GAG production demonstrated no significant difference between DPSCs and BM-MSCs after 21 days of differentiation (Figure 2.2D). Moreover, when the chondrogenic differentiation medium was supplemented with L-PRF ex or L-PRF CM, the percentage of the alcian blue-stained area in micropellets derived from both cell types was not significantly different (Figure 1D). Aggrecan expression could only be detected in differentiated BM-MSCs and remained absent in DPSC-derived pellets (Figure 2.2E, F). Likewise, when the differentiation period was extended to 28 and 35 days, no aggrecan expression could be detected in micropellets derived from DPSCs (data not shown). Exposure to L-PRF ex or CM did not significantly augment the aggrecan expression in cartilage spheres derived from BM-MSCs (Figure 2.2F). The control pellet resulted in 36.69% ± 10.89% aggrecan-positive stained area, while 5% L-PRF CM caused 27.24% ± 16.22% aggrecan-positive area compared to 21.34% ± 4.61% aggrecan-stained area in BM-MSCs supplemented with 25% L-PRF CM.

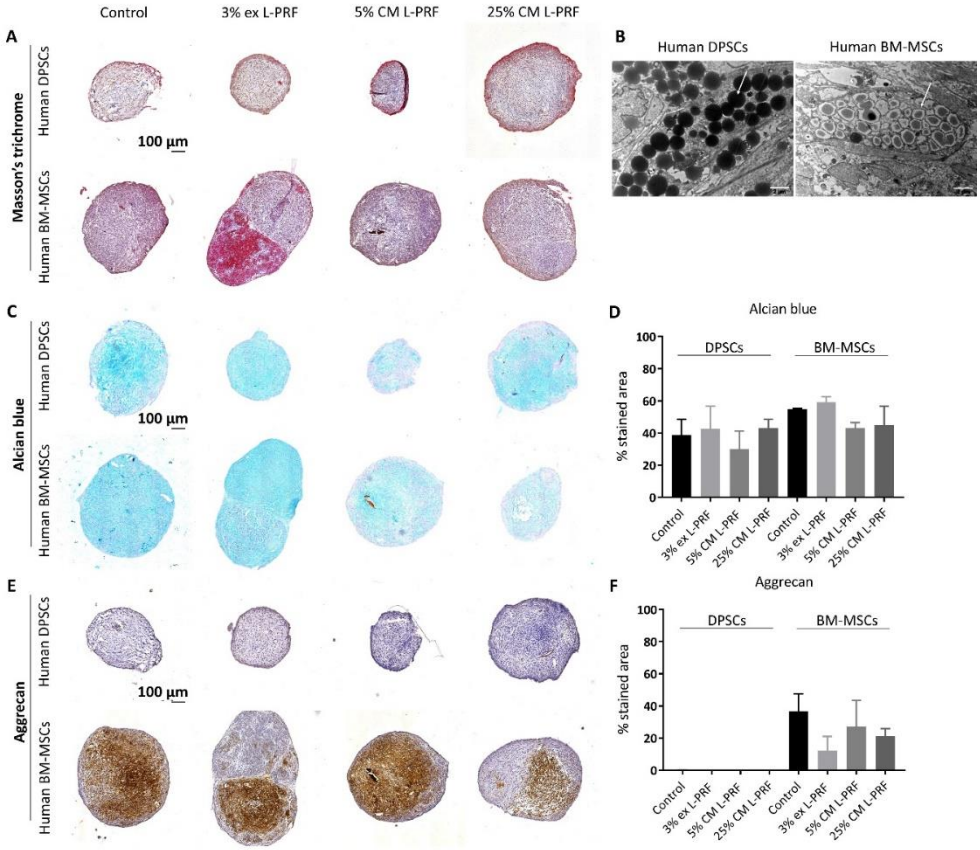

Figure 2.2. Differences in chondrogenic differentiation potential between human dental pulp stem cells (DPSCs) and human bone marrow-derived mesenchymal stem cells (BM-MSCs) and the effect of exposure to L-PRF during chondrogenesis. After 21 days of exposure to L-PRF ex (3%) or L-PRF conditioned medium (CM) (5% and 25%), cartilage-specific protein expression in differentiated pellets was evaluated using (immuno)histological staining (n = 3). (A) Masson's trichrome staining revealed the presence of abundant extracellular matrix (ECM) in micropellets derived from both differentiated stem cell types. (B) Ultrastructural analyses of the micropellets of both cell types showed the presence of dense matrix-filled vesicles (arrowheads). (C) Glycosaminoglycan (GAG) production was assessed by means of alcian blue staining. (D) L-PRF ex or L-PRF CM stimulation did not enhance the GAG area percentage. (E) Immunohistochemical (IHC) revealed that aggrecan expression was present in differentiated BM-MSCs, but absent in the DPSC-derived pellets. (F) Aggrecan area percentage was not enhanced by L-PRF ex or L-PRF CM exposure. Scale bars A, C, E = 100 µm; B = 2 µm. Data in D and F are represented as mean ± S.E.M.

2.4.2. Phenotypical and Ultrastructural Characterization of Immature Murine Articular Chondrocytes

iMACs were isolated from the femoral heads, femoral condyles and tibial plateau from hind limbs of 5–6-day-old wild type C57BL/6 mice. Phase contrast images revealed a rounded and polygonal morphology with a granular cytoplasm (Figure 2.3A). Expression of the main markers of chondrocyte phenotype was assessed via (immuno)histology. Alcian blue and toluidine blue staining show the presence of PG components (Figure 2.3B, C), while ICC demonstrated collagen type II expression by iMACs (Figure 2.3D). The average culture purity was 93.24% ± 1.33% (n = 3). Together, iMACs synthesise type II collagen and sulphated PGs *in vitro* after 4 days, showing the isolation of functional chondrocytes. Ultrastructurally, chondrocytes were characterised by a rounded, spherical morphology with ample rough endoplasmic reticulum, mitochondria and glycogen-rich vacuoles (Figure 2.3E and insert).

2.4.3. Effect of Secreted Factors of DPSCs and L-PRF on Healthy Chondrocyte Survival and Proliferation and Viability of TNF-α- and IL-1β-Stimulated iMACs

In order to evaluate the influence of L-PRF ex, L-PRF CM and DPSC CM on the viability of unstimulated or cytokine-stimulated iMACs, a PI test was employed at different time points (Figure 2.3F-K). After 24 h, serum deprivation decreased survival compared to iMACs cultured in high serum conditions (Figure 2.3F, I). This effect could not be prevented by supplementation of L-PRF ex to iMACs (Figure 2.3F). In contrast, the highest L-PRF CM concentrations (25% and 50%) had a significant pro-survival effect compared to the negative control condition and this was demonstrated to have a proliferative influence when serum was absent (139% ± 11.93% for 25% L-PRF CM and 120.2% ± 3.02% for 50% L-PRF CM) (Figure 2.3F). When 2% serum was supplemented, all L-PRF CM concentrations (5%, 25% and 50%) significantly increased iMAC proliferation at 48 h and 72 h (Figure 2.3G). When iMACs were stimulated with TNF-α and IL-1β, 25% and 50% L-PRF CM showed a statistically significant increased viability compared to the cytokine-stimulated negative control at 48 h and 72 h (177% ± 39.51% and 183.7% ± 38.24% for 25% and 50% L-PRF CM respectively compared to 65.1% ± 17.4% for the stimulated negative control for 48 h, 196.4%

± 33.86% and 231.2% ± 45.66% for 25% and 50% L-PRF CM respectively compared to 53.41% ± 26.7% for the stimulated negative control at 72 h) (Figure 2.3H). L-PRF ex did not exert any stimulating effects on proliferation or viability of neither unstimulated nor cytokine-stimulated iMACs. In serum-deficient conditions, DPSC CM significantly stimulated iMAC survival compared to the negative control (84.16% ± 12.06% compared to 49.08% ± 11.81%) after 24 h (Figure 2.3I). In 2% serum conditions, iMAC underwent a significant increased proliferation compared to the negative control after 48 h and 72 h when cultured in DPSC CM (Figure 2.3J). When cytokine-stimulation was implemented, iMAC viability followed an increasing trend when cultured in DPSC CM at every time point, although this effect was not significant (Figure 2.3K).

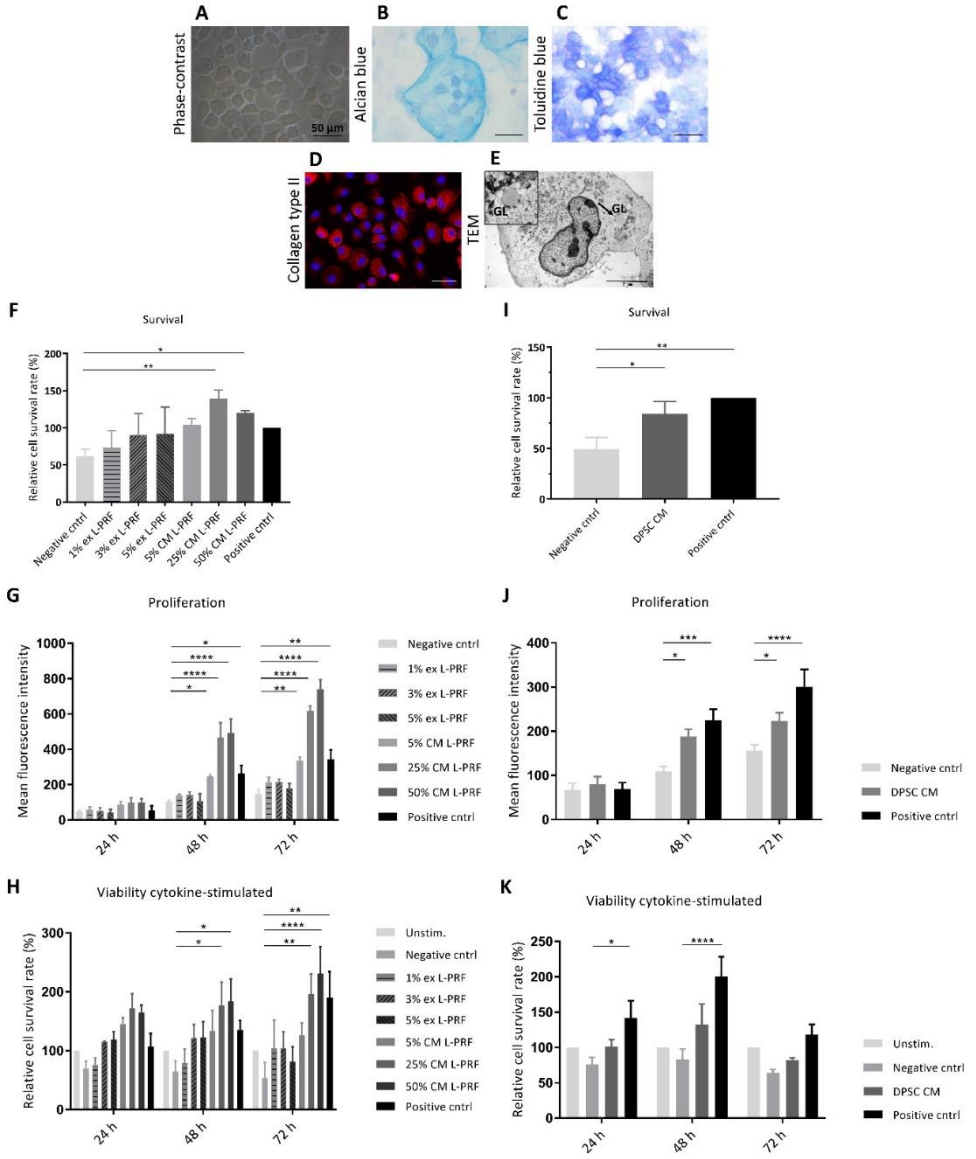

Figure 2.3. Phenotypic characterization of immature murine articular chondrocytes (iMACs) and the effect of L-PRF ex, L-PRF CM and DPSC CM on iMAC survival, proliferation and chondrocyte viability in TNF-α- and IL-1β-stimulated conditions. (A) Phase contrast micrographs of mouse iMACs show a rounded, polygonal morphology. (B-D) Histological staining revealed the production of sulphated PGs, while immunofluorescence staining demonstrated collagen type II expression. (E) Transmission electron microscopy (TEM) showed a rounded, spherical morphology with abundant rough endoplasmic reticulum, mitochondria and glycogen-rich (GL) vacuoles (insert). The effect of

the secretome of L-PRF and DPSCs on unstimulated iMAC survival, proliferation and cytokine-stimulated iMAC viability were evaluated by means of a PI assay (F-K). (F) 25% and 50% L-PRF CM had a significant pro-survival effect on iMACs after 24 h compared to the negative control (n = 5). (G) 5%, 25% and 50% L-PRF CM had a significant proliferative effect on iMACs after 48 h and 72 h compared to the negative control (n = 4). (H) 25% and 50% L-PRF CM significantly increased the viability of TNF-α- and IL-1β-stimulated iMACs after 48 h and 72 h (n = 5 for 24 h, n = 6 for 48 h, n = 6 for 72 h). (I) DPSC CM had a significant pro-survival effect on iMACs after 24 h compared to the negative control (n = 8). (J) DPSC CM significantly increased the proliferation of iMACs after 48 h and 72 h (n = 7 for 24 h, n = 8 for 48 h, n = 9 for 72 h). (K) TNF-α- and IL-1β-stimulated iMAC viability follows an increasing trend after exposure to DPSC CM, although not statistically significant. (n = 8 for 24 h, n = 10 for 48 h, n = 8 for 72 h). Scale bars A, B, C and D = 50 µm. Scale bar E: 5 µm (original magnification: 5,600). Data are represented as mean ± S.E.M. *. $p \leq 0.05$. **. $p \leq 0.01$. ***. $p \leq 0.001$. ****. $p \leq 0.0001$.

2.4.4. Effect of Secreted Factors of DPSCs and L-PRF on chondrogenic mRNA Expression of Unstimulated iMACs

Expression levels of chondrocyte-markers were investigated in unstimulated iMACs cultured with 3% L-PRF ex, 25% L-PRF CM and DPSC CM after 24 h (Figure 2.4). *Aggrecan* mRNA levels were significantly decreased upon supplementation of 3% L-PRF ex and 25% L-PRF CM (Figure 2.4A). 25% L-PRF CM significantly decreased mRNA levels of *collagen type II α 1* (Figure 2.4B). *TGF-β* mRNA levels were not significantly altered by L-PRF ex, L-PRF CM and DPSC CM (Figure 2.4C). Matrix metalloproteinase *(MMP)-13* was significantly upregulated in iMACs cultured with 25% L-PRF CM compared to control (Figure 2.4D), while *TIMP-1* mRNA expression levels were significantly upregulated by the supplementation of 25% L-PRF CM and DPSC CM (Figure 2.4E).

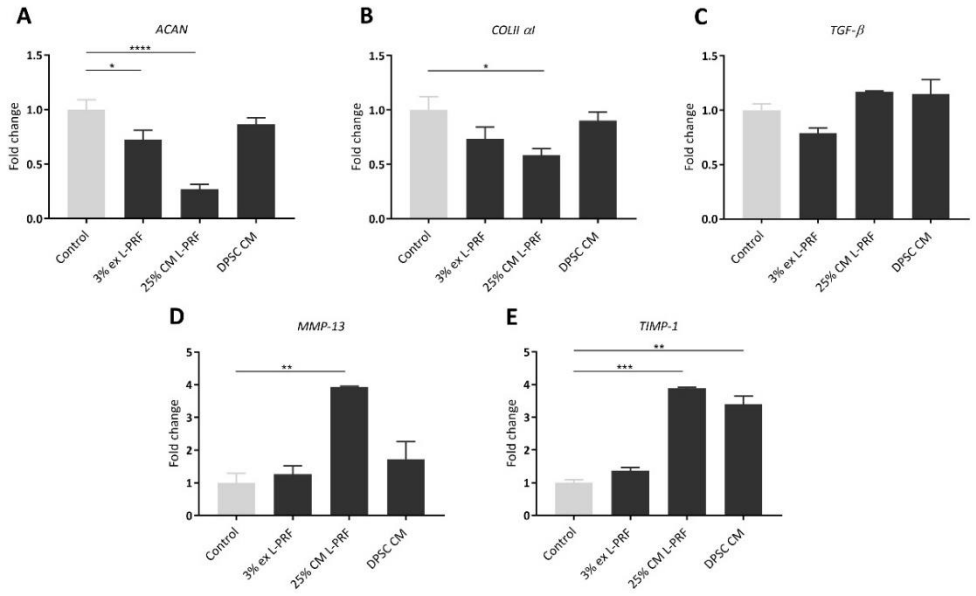

Figure 2.4. The effect of L-PRF ex, L-PRF CM and DPSC CM on chondrogenic genes of iMACs. Gene expression levels of chondrogenic markers were determined by RT-qPCR of unstimulated iMACs exposed to 3% L-PRF ex, 25% L-PRF CM and DPSC CM. (A-B) 25% L-PRF CM significantly decreased expression levels of collagen type II α 1 and aggrecan, while 3% L-PRF exudate only downregulated aggrecan expression levels. (C) TGF-β mRNA levels were not significantly altered by L-PRF ex, L-PRF CM and DPSC CM. (D) MMP-13 was significantly upregulated in iMACs cultured with 25% L-PRF CM compared to the control. (E) TIMP-1 mRNA expression levels were significantly upregulated by the supplementation of 25% L-PRF CM and DPSC CM. Data correspond to n = 6 for L-PRF ex and L-PRF CM, and n = 7 for DPSC CM. Data are represented as mean ± S.E.M. *. $p \leq 0.05$. **. $p \leq 0.01$. ***. $p \leq 0.001$. ****. $p \leq 0.0001$.

2.4.5. Effect of Secreted Factors of DPSCs and L-PRF on OA-related mRNA Expression of Unstimulated and TNF-α- and IL-1β-Stimulated iMACs

After iMACs were cytokine stimulated for 24 h and cultured in experimental conditions for another 24 h, gene expression levels of OA-related markers *aggrecan*, *collagen type II α 1*, *TGF-β*, *MMP-13*, *TIMP-1*, a disintegrin and metalloproteinase (*ADAM)-17*, *IL-6*, *TNF-α* and inducible nitric oxide synthase (*iNOS)* were measured. As shown in Figure 2.5, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) results showed that cytokine stimulation of

iMACs significantly decreased cartilage-specific mRNA levels, such as *aggrecan* and *collagen type II α 1*, compared to unstimulated iMACs after 24 h (Figure 2.5A, B). No significant increase in *aggrecan* or *collagen type II α 1* could be observed when iMACs were cultured with 3% L-PRF ex, 25% L-PRF CM or DPSC CM (Figure 2.5A, B). mRNA levels of *TGF-β*, a growth factor playing indispensable roles in cartilage integrity and homeostasis, were also measured using RT-qPCR and were not significantly altered (Figure 2.5C). TNF-α and IL-1β stimulation of iMACs increased mRNA levels of the chondrocyte maturation marker *MMP-13* compared to unstimulated iMACs (Figure 2.5D). Moreover, 25% L-PRF CM significantly increased *MMP-13* mRNA levels (Figure 2.5D), while *TIMP-1* was significantly upregulated by the supplementation of pro-inflammatory cytokines combined with 25% L-PRF CM and DPSC CM compared to the stimulated control after 24 h (Figure 2.5E). *ADAM-17* mRNA levels were significantly increased upon exposure to cytokines, but not altered by the supplementation of L-PRF ex, L-PRF CM or DPSC CM (Figure 2.5F). Cytokines with 25% L-PRF CM significantly amplified the *IL-6* mRNA levels compared to the stimulated control (Figure 2.5G). *TNF-α* and *iNOS* mRNA levels were significantly increased upon exposure to cytokines, and are not altered upon supplementation of L-PRF ex, L-PRF CM or DPSC CM (Figure 2.5H, I).

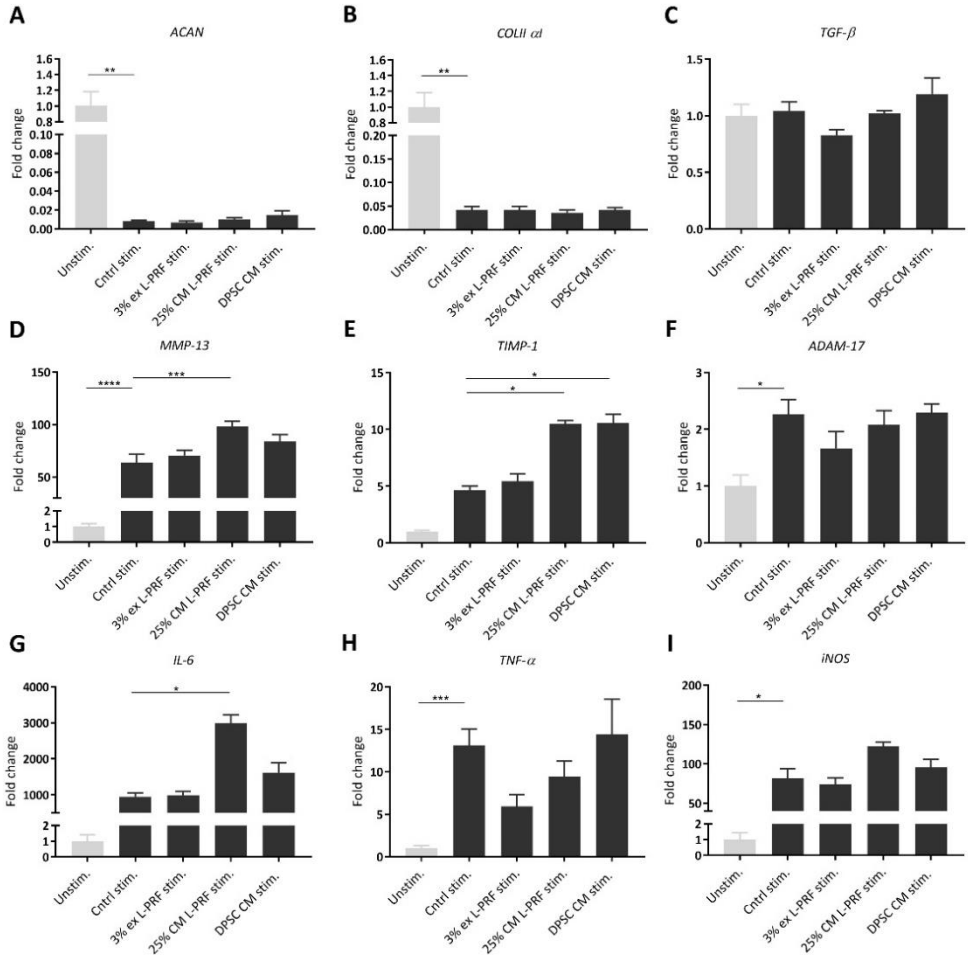

Figure 2.5. Effect of L-PRF ex, L-PRF CM and DPSC CM on TNF-α- and IL-1β-stimulated iMAC OA-related gene expression. Relative mRNA levels were determined by RT-qPCR of unstimulated and cytokine-stimulated iMACs exposed to 3% L-PRF ex, 25% L-PRF CM and DPSC CM. (A–B) Chondrocyte marker genes, aggrecan and collagen type II α 1, were significantly downregulated by cytokine stimulation, but not significantly altered by L-PRF ex, L-PRF CM and DPSC CM. (C) TGF-β mRNA levels were not altered upon exposure to cytokines, nor in combination with L-PRF ex, L-PRF CM or DPSC CM. (D) MMP-13 was significantly upregulated after cytokine stimulation, while 25% L-PRF CM further increased MMP-13 mRNA levels compared to the stimulated control. (E) TIMP-1 was upregulated by the supplementation of pro-inflammatory cytokines combined with 25% L-PRF CM and DPSC CM. (F) ADAM-17 expression was significantly upregulated after cytokine stimulation but not altered after exposure to L-PRF ex, L-PRF CM and DPSC CM. (G) 25% L-PRF CM significantly augmented the IL-6 mRNA levels compared to the stimulated control. (H–I) TNF-α and iNOS

mRNA levels were upregulated upon exposure to cytokines, but not altered by the supplementation of L-PRF ex, L-PRF CM or DPSC CM. Data correspond to n = 6 for L-PRF ex and L-PRF CM and n = 7 for DPSC CM. Data are represented as mean ± S.E.M. *. p ≤ 0.05. **. p ≤ 0.01. ***. p ≤ 0.001. ****. p ≤ 0.0001.

2.4.6. IL-6 and PGE2 Release Are Increased After Supplementation of Cytokines Combined With L-PRF CM

The medium of iMACs cultured in 3% L-PRF ex and 25% L-PRF CM and DPSC CM was collected after 24 h and subjected to an ELISA for IL-6 and PGE2 (Figure 2.6). Cytokine stimulation enhanced IL-6 production by iMACs, although not significantly, from 0 ng/mL for the unstimulated control to 6.31 ng/ml ± 1.65 ng/mL for the stimulated control (Figure 2.6A). Of all experimental conditions, only 25% L-PRF CM significantly enhanced IL-6 secretion (Figure 2.6A). Stimulation with cytokines in combination with 25% L-PRF CM induced a significant increase in PGE2 release by iMACs (86 ng/mL ± 24.14 ng/mL for stimulated 25% L-PRF CM compared to 2.75 ng/mL ± 1.11 ng/mL for the stimulated control) (Figure 2.6B).

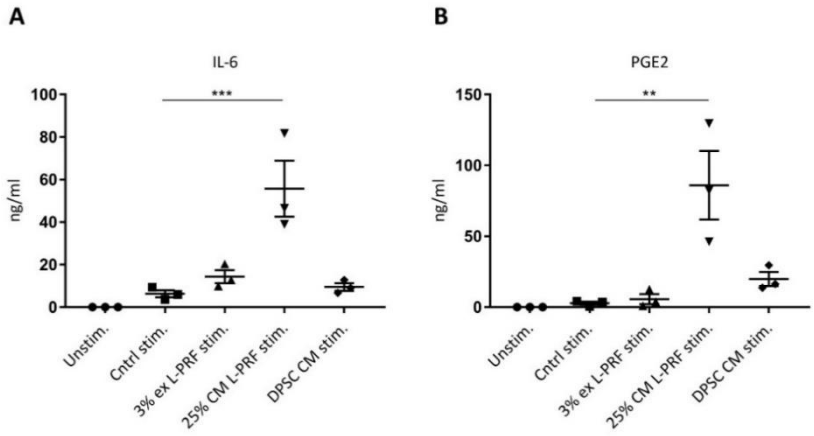

Figure 2.6. IL-6 and PGE2 secretion of iMACs after exposure to inflammatory cytokines and L-PRF ex, L-PRF CM and DPSC CM, measured via ELISA. (A) IL-6 release of iMACs is significantly increased after exposure to cytokine stimulation combined with 25% L-PRF CM. (B) Stimulation with cytokines in combination with 25% L-PRF CM induced a significant increase in PGE2 release by iMACs. Data correspond to n = 3. Data are represented as mean ± S.E.M. **. p ≤ 0.01. ***. p ≤ 0.001.

2.4.7. Nitrite Levels Are Increased Upon Cytokine Stimulation and Decreased by DPSC CM

To evaluate the influence of secreted factors of L-PRF and DPSCs on iMAC nitrite secretion, a Griess assay was performed. iMACs secreted significant more nitrite when they were stimulated with TNF-α and IL-1β in monolayer and 3D pellet culture (Figure 2.7A, B). In monolayer, 25% L-PRF CM exerted a small decrease in the nitrite secretion from 30.09 µM ± 1.69 µM to 26.69 µM ± 1.13 µM (Figure 2.7A). However, this effect was not significant. Also in pellet culture, L-PRF CM exerted a small not significant decrease in nitrite release by cytokine-stimulated iMACs. L-PRF ex did not decrease the nitrite secretion in cytokine-stimulated iMAC after 24 h in monolayer, nor after 72 h in pellet culture. CM of DPSCs induced a small, but not significant reduction in nitrite production of iMACs after 24 h from 30.09 µM ± 1.69 µM to 26.33 µM ± 1.84 µM in monolayer (Figure 2.7A). Remarkably, DPSC CM significantly decreased nitrite secretion of iMACs in micromass culture from 30.25 µM ± 1.87 µM to 17.08 µM ± 2.42 µM (Figure 2.7B).

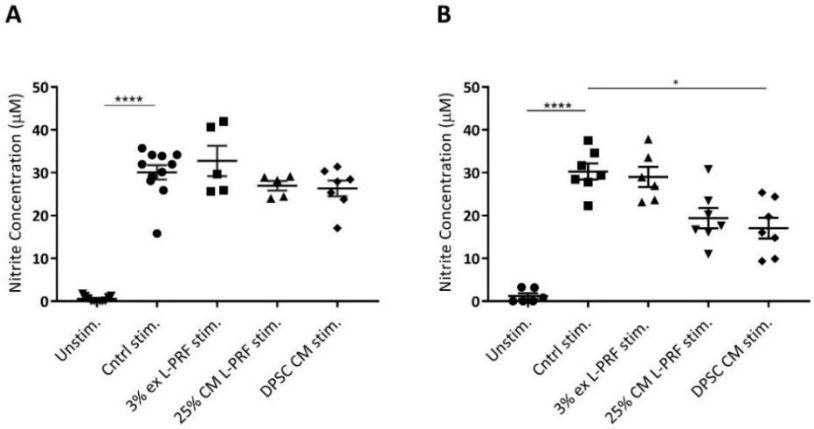

Figure 2.7. The effect of L-PRF ex, L-PRF CM and DPSC CM on TNF-α and IL-1β-stimulated iMAC nitrite release. Nitrite production in iMACs cultured in monolayer (A) and micropellet (B) was measured via the Griess assay. (A) In monolayer culture, nitrite production was significantly increased upon cytokine stimulation but not significantly altered by exposure to L-PRF ex, L-PRF CM and DPSC CM after 24 h. (B) In 3D micropellets, DPSC CM significantly reduced nitrite release of iMACs after 72 h. Data correspond to n = 5 for L-

PRF ex and L-PRF CM, n = 7 for DPSC CM (A), n = 6 for L-PRF ex, n = 7 for L-PRF CM and DPSC CM (B). Data are represented as mean ± S.E.M. *. $p \leq 0.05$. ****. $p \leq 0.0001$.

2.4.8. Cartilage-Specific ECM Production of iMACs in 3D Culture After Exposure to L-PRF ex, L-PRF CM and DPSC CM

To test the effect of secreted factors of L-PRF and DPSCs on the cartilage-matrix production of cytokine-stimulated iMACs, 5×10^5 iMACs cultured in micromasses were stimulated with TNF-α and IL-1β for 24 h. Afterwards, experimental conditions were added and 72 h later cell pellets were used for histological examination of the cartilaginous structure. Unstimulated iMAC pellets generated a typical cartilage-like tissue composed of chondrocytes in distinct lacunae surrounded by a dense PG-rich matrix as shown by representative images of alcian blue, toluidine blue and safranin O staining (Figure 2.8, arrowheads). However, pellets formed by cytokine-stimulated iMACs developed into a more fibrous tissue in which cartilage-lacunae were less evident and meaningfully decreased ECM and GAG production could be observed. This was revealed by an obvious decrease in alcian blue, toluidine blue and safranin O staining intensity (Figure 2.8). Cytokine-stimulated iMACs cultured with 3% L-PRF ex, 25% L-PRF CM and DPSC CM attained slightly more typical cartilage-like lacunae and showed a weak tendency of improved ECM content and chondrocyte status compared to the cytokine-stimulated control. A tendency to a higher alcian blue staining intensity could also be observed when cytokine-stimulated iMACs were cultured on L-PRF ex, L-PRF CM and DPSC CM.

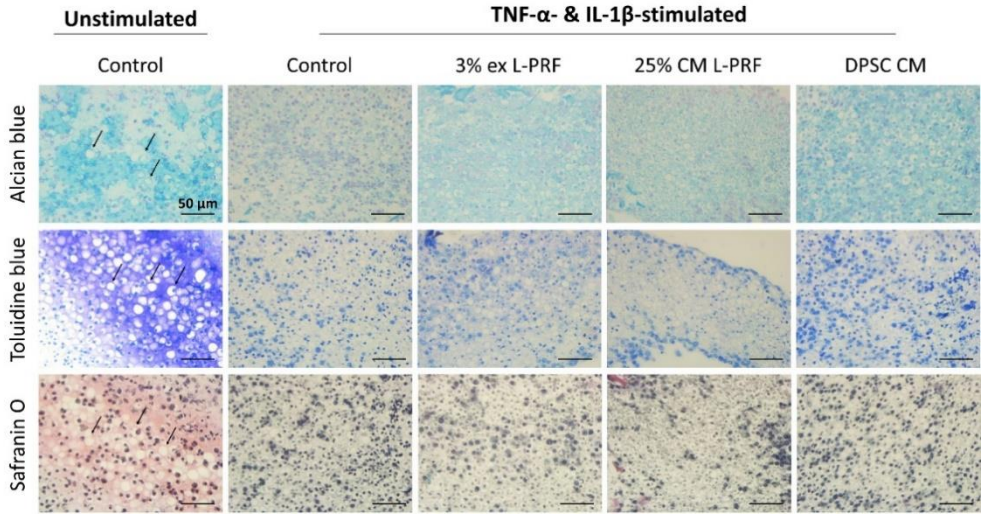

Figure 2.8. TNF-α- and IL-1β-stimulated iMACs cultured in 3D pellets attenuated a more cartilage-like morphology after exposure to L-PRF ex, L-PRF CM and DPSC CM. Representative images showed that unstimulated iMAC pellets generated a cartilage-like tissue with large numbers of chondrocytes present in lacunae (arrowheads). In the cytokine-stimulated control condition, iMACs developed into a more fibrous tissue in which cartilage-lacunae were less evident and GAG production is meaningfully reduced, as shown by an apparent decrease in alcian blue, toluidine blue and safranin O staining intensity. Cartilage lacunae were more preserved by L-PRF ex, L-PRF CM and DPSC CM with a weak tendency of improved ECM content and chondrocyte status compared to the stimulated control. Data correspond to n = 3. Scale bars = 50 µm.

2.4.9. Migration Capacity of Human DPSCs Towards Healthy iMACs

The chemoattractant properties of iMACs were evaluated by means of a transwell migration assay. Quantification of the migration area demonstrated the migration of DPSCs towards iMACs (Figure 2.9A). iMACs significantly attracted DPSCs after 24 h of incubation; 30.84% ± 11.5% migration area percentage of DPSCs compared to 1.89% ± 1.8% for the negative control.

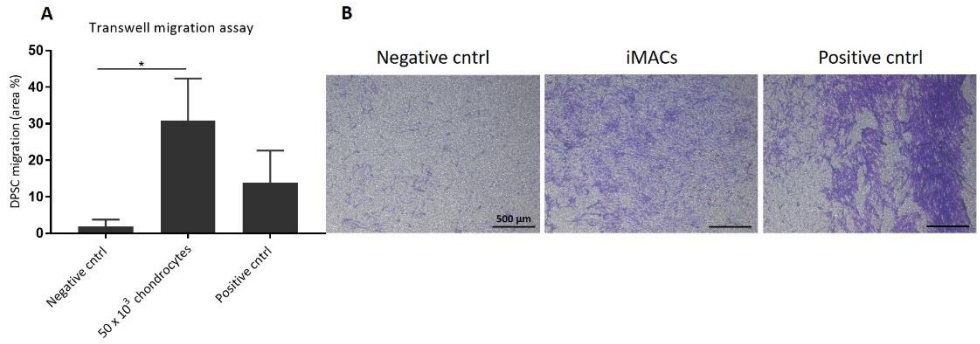

Figure 2.9. The migratory capacity of DPSCs towards iMACs after 24 h. The migration of DPSCs towards iMACs was evaluated using a transwell migration assay. (A) Quantification of the migration area demonstrated the migration of DPSCs towards iMACs (n = 5). (B) Representative pictures of the negative control, DPSC migration towards iMACs and positive control respectively. Scale bars = 500 µm. Data are represented as mean ± S.E.M. *. $p \leq 0.05$.

2.5. Discussion

The suggested mechanisms via which MSCs mediate cartilage repair and aid in OA include replacement of damaged cartilage tissue and paracrine-mediated effects such as proliferation of endogenous cells and immunomodulation (6).

In the first phase of the current study, the chondrogenic differentiation capacities of DPSCs were compared to BM-MSCs. Both BM-MSCs and DPSCs were shown to generate compact cartilage-like 3D spheres by differentiated cells surrounded by abundant ECM and GAGs. One of the most predominant PG, aggrecan, was not expressed in differentiated DPSCs, but cartilage spheres generated by BM-MSCs show abundant aggrecan secretion in the ECM. The absence of aggrecan in differentiated DPSC pellets in our study might be ascribed to several factors. One of these factors might be the differentiation time since an improved chondrocyte phenotype is reported upon prolonged culture times (226). After a differentiation period of 6 weeks, aggrecan expression was reported in human DPSCs by Mata and colleagues (144). However, Longoni *et al.* reported aggrecan expression after already 21 days of differentiation (227). Another possible explanation might be the used chondrogenic stimulus. The same research group stimulated cells by using TGF-β1, while the used chondrogenic stimulus in our study was TGF-β3 (227). Though both isoforms have been described to be key in chondrogenesis, the distinct isoforms might be involved in different stages of chondrogenesis. In posterofrontal suture derived MSCs, for example, TGF-β3 significantly increased proliferation of mesenchymal cells, while TGF-β1 is involved in mesenchymal cell condensation thereby stimulating differentiation (228). Another factor might be the culture settings as many utilised scaffolds to improve the phenotype of DPSC-derived chondrocytes *in vitro*, including hydrogels containing poly(ethylene glycol) dimethacrylate (PEGDMA), methacrylated gelatin (GelMA) and hyaluronic acid (HA) (145) and chitosan- based scaffolds (146), but did not always test for aggrecan expression. In addition, also hypoxic conditions and the addition of specific carbohydrates or growth factors might improve the expression of cartilage-specific components (154, 229, 230). Dai *et al.* reported that costal chondrocytes combined with exogenous FGF-9 are suitable to supply chondro-inductive stimuli to DPSCs (147). Rizk and colleagues showed that TGF-β3-transduced DPSCs express chondrogenic markers, including aggrecan (148).

Similar to our data, they showed that a positive staining for aggrecan was not evident in micromasses made by non-transduced DPSCs. Finally, the absence of aggrecan expression in our study might also have been influenced by inter-donor variability, as for example donor age might impact MSC differentiation (231, 232).

Though DPSCs generated a GAG and collagen-rich matrix, when compared to BM-MSCs, the lower GAG deposition and the absence of aggrecan suggest a differential chondrogenic potential. This might be associated with the fact that DPSCs are derived from the neural crest. In contrast to other bones of the body, which are derived from the mesoderm and ossify by endochondral ossification, bones from the craniofacial region originate from the neural crest and undergo intramembranous ossification during development (233). Moreover, neural crest cells are involved in the development of the temporomandibular joint (TMJ) and Meckel's cartilage, which consist of fibrocartilage and hyaline cartilage respectively (227). Therefore, future studies should focus on the type of cartilage and the type of collagens that are being deposited by DPSCs. The latter has been addressed in a report of Longoni *et al.*, in which they show that under various chondro-inductive conditions DPSCs formed more fibrocartilage-like tissues instead of hyaline cartilage (227).

Chondrogenesis of MSCs has been shown to be enhanced by the supplementation of growth factors (154). The beneficial properties of L-PRF have mainly been attributed to the high concentration of platelets, leukocytes and the long-term release of growth factors by the L-PRF matrix (234). We investigated the effect of L-PRF ex and L-PRF CM on the chondrogenic differentiation of DPSCs and BM-MSCs. Our results show that L-PRF ex and L-PRF CM were neither able to significantly increase the GAG secretion in both cell types nor induce aggrecan expression in DPSCs. Reports on chondro differentiation-promoting effects of platelets aggregates, such as PRP, on MSCs are contentious. Several previously confirmed chondro-inductive stimuli of platelet concentrates to MSCs (172, 235-237), whereas others indicate that PRP treatment does not improve the *in vitro* chondrogenesis of MSCs (238). The difference between the previously identified differentiation-promoting effects of platelets aggregates such as PRP in musculoskeletal diseases (reviewed by Qian *et al.* (239)), and our data on L-PRF might be caused by different factors. First, various platelet concentrates have

different release kinetics (240). Second, compared to other platelet concentrates, L-PRF contains significantly higher concentrations of leukocytes (161). With reference to this, the leukocytes in L-PRF have positive effects (e.g. anti-microbial properties (161)), but might at the same time be involved in catabolic pathways (241). Moreover, the leukocyte fraction in L-PRF has been reported to be accountable for the overproduction of several growth factors, including VEGF and inflammatory cytokines (161, 242), which have been described to negatively impact chondrogenesis *in vitro* (221, 243-248). In contrast, many other growth factors present in L-PRF ex and L-PRF CM are reported to have beneficial influences on MSC chondrogenesis (164, 221, 249, 250). To date, our data strongly indicate that the supplementation of L-PRF ex and L-PRF CM does not alter MSC chondrogenesis *in vitro*. Moreover, while most studies focus on replacing the chondrogenic stimulus by a platelet concentrate, we studied the additive effects of L-PRF on chondrogenesis. In our experiments, the chondrogenic stimulus was not replaced by L-PRF exudate or L-PRF CM, but various concentrations of L-PRF exudate or L-PRF CM were added to the complete differentiation medium, which might also explain no enhancing effects since high levels of growth factors might be deleterious for tissue formation (251). Studying the inductive effect of L-PRF rather than the additive effect on chondrogenesis might therefore be an alternative future experimental approach.

In a second phase, the secretome-mediated effects of human DPSCs and L-PRF on (TNF-α- and IL-1β-stimulated) iMACs were investigated. iMACs were isolated and phenotypically characterised based upon criteria identified by Gosset *et al.* (223). It is broadly documented that chondrocytes de-differentiate to fibroblast-like cells in monolayer and can bias outcomes (223, 252). To overcome this, all data were generated using freshly isolated chondrocytes. TNF-α- and IL-1β-stimulated chondrocytes transformed into cells with a reduced function, such as decreased cartilage-specific matrix mRNA levels, increased MMPs, inflammatory gene expressions and suppressed GAG production. These findings demonstrated the establishment of robust OA-mimicked chondrocytes *in vitro*.
We demonstrated that L-PRF CM significantly enhanced unstimulated iMAC survival, proliferation and TNF-α- and IL-1β-stimulated iMAC viability in a concentration-dependent manner. These effects were not observed in iMACs

cultured in the presence of L-PRF ex. In contrast to our findings, Chien *et al.* demonstrated that the exudate of PRF could improve chondrocyte proliferation when cultured in fibrin-based scaffolds (169). This alteration in outcome between the two L-PRF derivatives might be explained in the difference in growth factor levels. Specifically, significantly higher levels of growth factors are found in L-PRF CM compared to L-PRF ex, which can be caused by the fact that L-PRF CM is generated after incubation for 96 h, resulting in a continuous release of growth factors by the leukocytes in the fibrin matrix of the L-PRF (159, 221). RT-qPCR data demonstrated at 24 h post-stimulation a significant decrease in *aggrecan* and *collagen type II α 1* mRNA levels of healthy iMACs when cultured in the presence of L-PRF CM. L-PRF ex significantly decreased *aggrecan* mRNA expression. In addition, *MMP-13* and *TIMP-1* mRNA expressions were increased in unstimulated iMACs upon 25% L-PRF stimulation. The increased proliferative state of iMACs upon L-PRF CM supplementation seems to be accompanied by a downregulation of cartilage-specific ECM components and the upregulation of *MMP-13* in healthy iMACs. When iMACs were cytokine-stimulated, L-PRF CM significantly increased *MMP-13*, *TIMP-1* and *IL-6* mRNA levels. ELISA demonstrated a significant increase of IL-6 and PGE2 secretion, two inflammatory mediators in OA by cytokine-stimulated iMACs upon exposure to 25% L-PRF CM. IL-6 is widely known to mediate several pro-inflammatory responses contributing to the pathogenesis of several immune-related diseases, such as RA (253). Therefore, therapeutic targeting IL-6 has become important in the drug development applications of these diseases. Tocilizumab (TCZ), an IL-6 receptor-inhibiting monoclonal antibody, is widely used in the treatment of RA (254). However, the role of IL-6 in OA remains unclear. High levels of IL-6 are found in the synovial fluid of OA patients. These high IL-6 levels are associated with increased MMP levels and radiographic OA changes (255, 256). Additionally, it was reported that inhibition of IL-6 with TCZ lowered pain behaviour in an experimental model of OA in rats (257). In contrast, IL-6 knockout mice revealed the progression of more advanced OA than wild-type animals and injection of IL-6 in the joint of IL-6-deficient mice reduced cartilage loss during arthritis (258, 259). Nevertheless, based on the above outcomes, our data might indicate an inability of L-PRF to counteract cytokine-induced phenotypical changes of iMACs *in vitro*.

Several growth factors, such as VEGF, EGF, IL-6 and MCP-1, are highly present in L-PRF CM and in minor levels in the exudate (221, 245) and might be accountable for the observed effects in the present study. For example, VEGF is reported to act as a survival factor in growth plate chondrocytes and has proliferative effects in immortalised chondrocytes (260). Moreover, increased MMP levels and secretion are reported because of VEGF (261, 262). Pufe and colleagues also showed pro-inflammatory factors such as IL-1β, nitric oxide, TNF-α and IL-6 to be induced by VEGF (262). Controversies concerning the impact of VEGF in cartilage repair and OA are stated. Hypoxia is needed to maintain proper chondrocyte phenotype. Via VEGF, this hypoxic state is reduced through increased vasculature, resulting in osteogenic-differentiating stimuli to form bone cells from chondrocytes (263). On the other hand, blocking VEGF was shown to inhibit chondroprogenitor cell proliferation and migration *in vitro*. Also, the complete inactivation of VEGF-A in areas of collagen type IIα1 expression resulted in embryonic lethality. These data indicate that a strictly controlled VEGF expression is indispensable for limb development, and thus chondrogenesis (263). Also EGF and IL-6 increased numbers of chondrocytes (264, 265). Furthermore, IL-6 is described to be able to increase MMP expression alone or in synergy with IL-1β and oncostatin M (266-268). In addition, MCP-1 increased MMP-13 expression in chondrocytes (269). Furthermore, several other proteins that are abundantly present in L-PRF CM, such as RANTES, growth regulated oncogene (GRO) and IL-8 might be responsible for the observed effects in our study (221). RANTES is demonstrated to induce chondrocyte expression of iNOS, IL-6 and MMP-1 (270), while IL-8 and GROα are shown to induce articular chondrocyte hypertrophy and calcification through increased type X collagen, MMP-13 expression and alkaline phosphatase activity (271).

In contrast to our findings, numerous other studies demonstrated that a large number of growth factors found to be secreted by platelet derivatives have predominantly beneficial and promising activities for (pre-)clinical applications for chondrogenesis and anti-inflammatory effects (165). To date, studies mainly focused on the role of platelets and platelet-derived growth factors, since these are the common features between all types of platelet concentrates, while future research should focus on identifying the role of the leukocytes and leukocyte-derived growth factors and cytokines in L-PRF. To our knowledge, reports on the

secretome-mediated effects of L-PRF on chondrocytes *in vitro* are limited. Injectable-PRF, generated by a low speed centrifugation approach, was found to counteract IL-1β inflammatory effects in chondrocytes (170). In addition, Wong *et al.* treated chondrocytes with different concentrations of PRF CM and showed a proliferative effect on chondrocytes and induced chondrogenic differentiation of chondrocytes (171). Moreover, Barbon *et al.* revealed that preclinical studies strongly indicate a significant enhancement of cartilage regeneration after PRF treatment (164). There are several reasons for the discrepancy in outcomes between our study and studies proving beneficial effects of PRF in OA. First of all, the L-PRF used in our study was human-derived, while iMACs were from murine origin. This might have played a role in the inflamed and hypertrophic phenotype of iMACs cultured in the presence of inflammatory cytokines and L-PRF exudate or CM. Secondly, previous reports used a different form of PRF or employed an alternative differentiation protocol. One study used injectable PRF, which is produced by low-speed centrifugation (170). Wong *et al.* generated L-PRF CM by putting L-PRF clots, that were previously frozen, in medium for 24 h at 4 °C (171). Moreover, the exudate and CM fractions used in our study were centrifuged and filtered in order to be acellular, which has not been specified in other reports. Finally, inter-donor variability might be a crucial factor.

In the present study, we show that the CM of DPSCs significantly enhances iMAC survival and proliferation *in vitro*. DPSC CM exerts the same, but smaller, effects on TNF-α and IL-1β-stimulated iMAC viability although not reaching statistical significance. DPSCs secrete various growth factors and cytokines, which might be accountable for the observed outcomes in the present study. Previous studies revealed high expression levels of TGFs and neurotrophic factors, including VEGF (219, 272, 273). Other factors present in the DPSC secretome involve but are not limited to IL-8, MCP-1, FGFs, MMPs, TIMP-1 (217, 219, 274). The presence of large quantities of VEGF in DPSCs could predominantly be responsible for the proliferative effects on iMACs (260). Narcisi *et al.* report that TGF-β1-stimulated chondrocytes evidenced increased mRNA levels for several hypertrophy-specific markers, including *MMP-13*, *VEGF* and *TIMP-3* (275). In our study, RT-qPCR data show that DPSC CM induced significantly increased *TIMP-1* expression in stimulated iMACs. TIMP-1 directly inhibits the activities of MMPs, thereby contributing to reducing the impact of MMPs (276, 277). Next to this, in cytokine-

stimulated chondrocytes cultured on DPSC CM a not significant trend towards increased PGE2 production was observed. The role of PGE2 is controversial in OA; though PGE2 exerts catabolic functions in OA, one of the main effectors of MSC-mediated immune-suppression is PGE2 (108).

Cartilage-specific ECM production of iMACs in 3D culture after exposure to the secretome of L-PRF and DPSCs was also evaluated. The benefit of using these micromass cultures compared to monolayer cultures is that the 3D setting is more representative of the *in vivo* microenvironment. In consistence with our RT-qPCR results, cytokine stimulation of iMACs induced meaningfully less production of PGs and GAGs in micromass cultures, accompanied by increased nitrite secretion. Cartilage lacunae were more preserved by 3% L-PRF ex, 25% L-PRF CM and DPSC CM with a weak tendency of improved ECM content and chondrocyte status as compared to the stimulated control. Moreover, DPSC CM significantly decreased nitrite levels of iMACs cultured in 3D micromasses.

It should be noted that the experiments in this study were conducted between 24-72 h post-stimulation, a time window in which the outcome on matrix components like aggrecan or collagen type II production is not yet observed. Therefore, we also evaluated fast acting proteins such as nitrite and PGE2. Nonetheless, subsequent studies are necessary to investigate the impact on the structural level of cartilage by means of longer *in vitro* cultures, cartilage-explant studies and *in vivo* experiments. Moreover the use of the chosen GAG quantification method might be an important limiting factor. No quantitative analysis of GAG composition was performed via, for example, the dimethyl-methylene blue (DMMB) assay, which is one of the most available techniques to assess tissue composition and allows for normalization to DNA content. However, since the DMMB assay does not allow for the combination of visualizing both histological characteristics and GAG quantification in the same sample, quantification of histological stains was preferred.

Concerning preclinical animal studies and since DPSCs show beneficial paracrine-mediated impacts on OA, an IA injection of DPSCs might be of particular interest. With respect to this, we evaluated the migratory capacity of human DPSCs towards iMACs by means of a transwell migration assay and demonstrated that human DPSCs were able to successfully migrate towards iMACs.

Given the pathophysiology of OA, the role of immune cells, other cell types present in the synovial joint and synovial joint structures should ideally also be taken into account. Therefore, in order to supply a proper *in vitro* OA model, the interchange of immune cells, the synovial membrane and subchondral bone with the cartilage tissue and chondrocytes needs to be addressed. One-dimensional cell culture models cannot fully mimic the complexity of the OA pathophysiology. However, several advantages are associated with monolayer or one-dimensional cell cultures such as a large number of cells can be easily isolated, and cells in monolayer permit the homogenous spread of cytokines and nutrients. Still, co-cultures or 3D cultures permit the study of cell-specific changes and cell–cell communications, while explant models inform on the induced alterations occurring in the ECM. The co-culture of the synovium with chondrocytes is one way to reproduce the complexity of the pro-inflammatory events *in vitro*. The use of bone in co-culture experiments is also crucial (278). Haltmayer *et al.* utilised a co-culture system with all three principal tissues involved in OA, such as cartilage, subchondral bone and the synovium (279).

2.6. Conclusion

The present study aimed to investigate the chondrogenic potential of both L-PRF and DPSCs *in vitro* in terms of being able to replace lost cartilage tissue, while having chondroprotective and immunomodulatory influences in OA chondrocytes. We show a discrepancy between BM-MSCs and DPSCs to form neo hyaline cartilage *in vitro* and that L-PRF did not improve or impede the chondrogenic differentiation of both DPSCs and BM-MSCs. However, DPSCs generated a GAG- and collagen-rich matrix, demonstrating that DPSCs are a promising cell source to make cartilage regeneration achievable. L-PRF CM exerted significant pro-survival and proliferative effects on chondrocytes and increased several inflammation-related mediators involved in OA. Nevertheless, transformation into hypertrophic chondrocytes remains an important matter that needs to be further elucidated. Our data show promising therapeutic effects of DPSCs to repair cartilage lesions and in an *in vitro* model mimicking OA, as they can potentially replace the damaged cartilage tissue and act via secretome-mediated effects. On the one hand, DPSC CM can stimulate endogenous cells to proliferate and replace the lost tissue, while on the other hand, it could prevent the progression of cartilage loss by impairing chondrocyte apoptosis. Moreover, we indicate that factors secreted by DPSCs might cause multiple anti-inflammatory and anti-catabolic influences in OA chondrocytes. In addition, the migratory capacity of human DPSCs towards chondrocytes might be essential in allowing IA injection of stem cells in future applications. Insights in the paracrine effects of DPSCs and understanding stem cell modulation will offer researchers a number of treatment options for musculoskeletal diseases and traumatic injury that have until now been limited by cell sourcing concerns. Finally, the influence of secretome-mediated actions of L-PRF and DPSCs on OA chondrocytes and other types of cells or joint structures involved in OA should additionally be investigated in longer-term co-culture systems or 3D cell culture settings. Furthermore, since hypertrophic chondrocytes are important in pathological modifications in OA, a future study to investigate the dedifferentiated or hypertrophic state of chondrocytes is warranted.

Chapter 4: Stem Cells for Cartilage Repair: Preclinical Studies and Insights in Translational Animal Models and Outcome Measures

Based on;

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4.1. Abstract

Due to the restricted intrinsic capacity of resident chondrocytes to regenerate the lost cartilage post-injury, stem cell-based therapies have been proposed as a novel therapeutic approach for cartilage repair. Moreover, stem cell-based therapies using mesenchymal stem cells (MSCs) or induced pluripotent stem cells (iPSCs) have been used successfully in preclinical and clinical settings. But before novel cell-based therapies for cartilage repair can be introduced into the clinic, rigorous testing in preclinical animal models is required. Preclinical models used in regenerative cartilage studies include murine, lapine, caprine, ovine, porcine, canine, and equine models, each associated with their specific advantages and limitations. The following chapter presents an overview of the advantages and disadvantages of utilizing small and large animals, while also describing suitable outcome measures for evaluating cartilage repair.

4.2. The Importance of a Translational Animal Model and Appropriate Outcome Measures

While *in vitro* studies and models offer a substantial amount of information about the potential of stem cells for cartilage repair (308, 309), more in-depth knowledge about their behavior *in vivo* should be derived from immunocompetent animal models. In orthopaedic research, to move new technologies from bench to bedside, strict preclinical studies using translational animal models are required (310). Preclinical studies evaluating the healing of cartilage defects have been performed using both small and large animal models including murine, lapine, porcine, caprine, ovine, canine and equine models (63, 311). The following section will focus on the advantages and disadvantages of utilizing small and large animals for cartilage repair studies as well as some key factors in study design and the usage of validated outcome measures.

4.3. Choice of Animal Model: Small Versus Large Animal Models

Articular cartilage defects have been created in small animals, such as mice (91), rats (312-315) and rabbits (316-318). Smaller animal models are cost-effective and easy to house and rodents are available in a variety of genetically modified strains with minimal biological variability (17, 311). However, the small joint size, the thin cartilage (319, 320), altered biomechanics (321, 322), and increased spontaneous intrinsic healing (323) hamper the study of the regenerative capacity of stem cells and these mechanisms of healing cannot be fully extrapolated to human cartilage repair (17, 311). Rodents have mainly been used to assess chondrogenesis of cell-based therapies by subcutaneous (324), intramuscular (325), and intra-articular (IA) (326) implantations of cells (17). Of all small animals, the rabbit model is the most utilized model in cartilage regeneration studies because of the slightly larger knee joint size in comparison to rodents (63). Despite their limited translational capacity, small animals can be very useful as a proof-of-principle study and to assess therapy safety before moving on to preclinical studies using larger animals (17, 310).

Large animal models play a more substantial role in translational research because of a larger joint size and thicker cartilage, however, their preclinical use is often hindered by high costs and difficulties in animal handling. A variety of large animal models have been used to investigate cartilage repair strategies, including horses (327-329), dogs (330), sheep (331-335), goats (336, 337) and (mini)-pigs (338-341), each with their own strengths and limitations.

The knee anatomy (342-344), cartilage thickness (319, 345), biomechanical loading environment (311) and the subchondral bone properties (322) of the above-mentioned species differ variously from the human condition (311, 346). An advantage of using the porcine model is the cartilage thickness of 1.5 mm-2 mm, compared to human cartilage thickness of 2.4 mm-2.6 mm (338, 345). Dogs, in contrast, have thinner cartilage (0.95 mm-1.3 mm) compared to human cartilage (311, 345). For the goat, cartilage thickness has been reported between 0.8 mm and 2 mm, whereas cartilage thickness in sheep ranges from 0.4 mm to 1.7 mm (311, 345). Of all animal models used in cartilage regeneration studies, the horse's cartilage thickness (1.75 mm-2 mm) provides the closest approximation to the human situation (319, 322, 345, 347).

In a comparative anatomical analysis, the goat stifle displayed strong anatomic similarities to the human knee except for a long trochlear groove with medial and lateral ridges and the intercondylar notch width (311, 342). According to Osterhoff *et al*., the ovine stifle is very similar to the human knee except for the femoral intercondylar notch width, the patellofemoral joint's biomechanics and the proximal tibia's cortical bone stock (344). More recently, Vandeweerd and colleagues described several anatomical features in the ovine stifle (343). Although the goat and ovine stifle are very similar to the human knee, these few anatomical differences remain and should be taken under consideration when selecting them as a suitable animal model (342-344), which, for instance, can have an impact on the volume of the synovial cavity. In addition to similar knee anatomy, the caprine model has been reported to have similar stifle biomechanics compared to human knees (311, 348). While the horse model offers defect sizes comparable to human defect dimensions, the increased weight and the fact that the horse spends much of its time in standing position place defects under significant loading and this continuous loading cannot be diminished (345).

Nevertheless, this constant loading environment in the horse stifle joint could be argued to be beneficial for translational cartilage repair studies since the human knee provides a less challenging load environment (349). Alternatively, ponies offer a good model; they closely mimic the human joint size and, in contrast to horses, have similar loads as humans (350).

Moreover, since numerous repair strategies rely on the subchondral repair mechanisms, subchondral bone properties must be considered when selecting the appropriate repair model (322). According to Chevrier *et al.,* the subchondral properties of the rabbit trochlea are similar to the human medial femoral condyle (MFC) (322). The goat offers advantages in subchondral bone consistency, thickness and trabecular structure, which are more similar to the human structure in comparison to either small animals, ovine or canine models (17, 311). A major disadvantage of the ovine and equine models is the dense and hard subchondral bone, while the caprine model has a softer subchondral bone (17, 345). In addition, subchondral bone cysts in sheep (331, 351) and goat (352) have been reported when the subchondral bone is involved in cartilage repair mechanisms (353).

Ultimately, when selecting the best repair model, comparable anatomy and joint function are not the only important aspects, but other factors need to be taken into consideration when performing translational preclinical studies (Table 4.1). A factor requiring major consideration is the choice of defect location (311). Clinically, most defects are made on the femoral condyles or the trochlear groove (346). However, defect position influences cartilage repair response as demonstrated in caprine and ovine models leading to contradictory results (333, 348). These differences in repair potential are due to differences in cartilage thickness, loading mechanics and subchondral bone properties within the knee and between species (322, 333, 348). In addition, defects may occur where higher loads are expected (354). Ideally, these areas should be used when defects are induced. Therefore, it is important to identify the prevalence of naturally occurring defects in animal models and to assess where the lesion should be created based on the biomechanics of the joint of the animal (311, 354). The ovine model is a well-documented model, where the most frequent naturally occurring cartilage defects in the ovine knee occur on the axial aspect of medial tibial condyle (MTC)

and on the MFC (354). Critical size chondral and osteochondral defects have been reported in rats, rabbits, dogs, (mini-)pigs, sheep, goats and horses (as shown in (310, 345, 355)). Skeletal maturity and animal age also affect repair mechanisms of cartilage defects, especially when the subchondral bone is fractured for induction of repair (322, 323, 353, 356, 357). Experimental models in animals that have reached skeletal and articular cartilage maturity are needed before the effect of any novel regenerative strategies on adult cartilage repair can be clinically evaluated. According to the International Cartilage Repair Society (ICRS) recommendations, selection of the age of an experimental animal should be based on cartilage maturity rather than on skeletal maturity (closure of the growth plate) (353). Cartilage maturity can be defined as the time point where a cartilage defect is not spontaneously repaired and at presence of a well-defined zonal architecture, an intact continuous layer of calcified cartilage, and minimal vascular penetration in the subchondral bone plate (353). This would confirm that the articular cartilage has the adequate cellular, biomechanical and biochemical properties. Therefore, in preclinical cartilage repair studies, animals at the age of cartilage maturity, defined based on the aforementioned conditions, should be used (Table 4.1) (353).

While the choice of animal age, critical defect dimensions and location in preclinical studies is often justified, gender selection is frequently overlooked. Regenerative strategies to address cartilage lesions and osteoarthritis (OA) have not sufficiently considered possible gender differences (358). Therefore, potential gender effects must be taken more into consideration during analysis. Epidemiological studies demonstrated the presence of sex differences in OA prevalence and incidence with females being at a higher risk to develop more severe knee OA after reaching menopausal age (358). Several researchers examined the role of sex hormones in OA, including in ovine and murine models (354, 359-361). Ma and colleagues showed that sex hormones, both testosterone and oestrogen, have a crucial influence on the advancement of OA in mice. Testosterone aggravated the disease in male mice evidenced by the fact that orchiectomized mice showed a less severe OA than intact males. Healthy female mice showed less severe OA than ovariectomized females, demonstrating the protective role of female hormones (361). In a biomechanical study in sheep, ovariectomy in females induced a detrimental effect on the intrinsic properties of the articular cartilage in the knee

(359). In human subjects, differences in knee joint volume and articular surface areas between men and women have been described (362). Moreover, gender differences in cartilage composition and gait mechanics in young healthy, middle-aged healthy, and OA cohorts are reported (363). These differences might influence functional outcome after repair (364). Thus, effective and well-designed regenerative preclinical studies are required and should lead to a better understanding of gender-specific differences in the mechanisms involved in cartilage re- and degeneration. Since OA and cartilage biology are reported to be sex-dependent, the inclusion of female animals is essential for preclinical cartilage repair studies. If both sexes are included, an equal number of males and females per study group with short ranges of ages should be used. Moreover, results should be reported for both genders and per study group (358). In addition, for large animals, it is more difficult to manage male animals, since sexual behaviour and mounting may increase loads on hind limbs.

Obviously, the recommended study duration for evaluating cartilage repair in preclinical animal models is different for proof-of-concept or pilot studies (< 6 months) versus late stage preclinical studies in large animal models (> 6 months) (310, 311, 353). However, for late stage preclinical studies, caution must be exercised when the study ends within a year or when no interval follow-up investigations are implemented since the repaired tissue can vary at earlier phases of healing and the sustainability of the repaired tissue is time-dependent (334, 339, 353). Follow-up methods of noninvasive imaging are necessary (365, 366). Ovine models allow for imaging techniques such as magnetic resonance imaging (MRI) (353, 367), while the equine model is much more difficult, or impossible, due to size of animal versus size and costs of high-field MRI. Furthermore, the nature of the regenerative strategy, such as the use of autologous or allogeneic cell therapy, also needs to be considered. Both approaches have their own shortcomings. If autologous treatment includes site morbidity and logistic problems, allogeneic use forms an important risk factor for immune rejection or transmission of disease (368). Particularly, considering the differential influence of autologous, allogeneic or xenogeneic cells on the immune system and the impact on tissue repair (369), when aiming the development of tissue engineered constructs for cartilage repair, it is essential to consider the impact of the tested treatment on the immune response.

Other key issues in cartilage repair models are the choice of bilateral versus unilateral surgery and acute versus chronic defects (334, 353). Bilateral repair models are suitable to minimize inter-animal variability and to increase the number of treated limbs, but are only useful if the treatments are not reciprocally influencing the opposite limbs (370). Unilateral models, in contrast, ensure that the treatment is not influenced by the contralateral technique. In addition, these models allow easier joint immobilization and are exposed to less initial weight bearing on the operated limb. More importantly, unilateral models permit better evaluation of locomotion, range of motion and gait (353).

Concerning the recommendations from regulatory bodies, the Food and Drug Administration (FDA), for example, has established that no perfect animal model exists for articular cartilage injury and both small and large animals should be included to assess safety, efficacy and durability of a treatment. However, they advise the use of large animals such as goats, sheep, and horses as suitable preclinical models (371). Moreover, various recommendation documents for preclinical cartilage repair studies are published by the FDA, European Medicines Agency (EMA), as well as the American Society for Testing and Materials (ASTM) International and ICRS and should preferably be applied. They provide a list of details for appropriate preclinical animal studies including commendations on study duration, lesion site, lesion location, use of cells and appropriate outcome measures (372). However, since these documents are not mandates but only offer advice, investigators can ultimately decide on the proper study design (372).

The choice of animal model is also influenced by practical aspects such as ethical considerations, costs and availability of housing accommodations, materials and competent personnel (346). Nowadays, it is increasingly difficult to obtain ethical permission for the usage of dogs and horses, while working with reformed sheep or goats is less hindered by ethical rejection. Surgical limitations, such as the ability of the animal to tolerate anaesthesia and post-surgical recovery protocols or the possibility of second-look access, could influence the choice of a specific animal model (327, 328, 353, 373). The ovine model, for instance, is particularly easy to handle, cost-effective and easy to anaesthetize.

Table 4.1. Key factors for the selection of a translational animal model for cartilage repair.

Aspect	Remark/Recommendation
Anatomy and biomechanics	▪ Large difference in anatomy and biomechanics remain between animal models and humans
Cartilage thickness	▪ Large animals provide closer proximity to the human condition ▪ Depends on topographic location in joint
Subchondral bone properties	▪ Effect on repair mechanisms ▪ Depends on topographic location in joint
Defect dimensions and location	▪ Critical size chondral or osteochondral ▪ Location of defect influences cartilage repair ▪ Femoral condyles or trochlea ▪ Defect should be made based on the biomechanics of the joint of the animal
Age and gender	▪ Age and gender may have effect on repair mechanism ▪ Inclusion of skeletally mature animals with mature cartilage (human-near puberty): - Rat-13 weeks - Rabbit-8 months - Dog-24 months - Pig-18 months - Sheep-24 months - Goat-24 months - Horse-24 months ▪ Gender effects must be taken into consideration ▪ Use animals with short range of ages and with similar sex
Study duration	▪ Depends on type of study ▪ Proof-of-principle (< 6 months) versus late stage study (6 months - 12 months)
Surgical and practical considerations	▪ Unilateral versus bilateral repair models - Unilateral models: evaluation of locomotion, range of motion and gait, better immobilization, no influence of contralateral technique - Bilateral models: minimize inter-animal variability ▪ Postoperative management should be tolerated ▪ Ethical permission for small animals and ruminants is easier to obtain ▪ Surgical feasibility must be taken into account ▪ Financial costs to house and handle differ variously between animals ▪ Availability of facilities, competent personnel and equipment
Validated outcome measures	▪ At baseline, *in vivo* and post mortem ▪ Clinical response and kinematics ▪ Biological fluid collection ▪ Noninvasive compositional imaging MRI

	▪ Ex vivo high resolution magnetic resonance imaging (MRI) or micro computed tomography (μCT) ▪ Tracking and monitoring ▪ Macroscopic/arthroscopic scoring ▪ Histological and histomorphometric scoring ▪ Mechanical testing ▪ Biomolecular and biochemical testing

4.4. Follow-up and Outcome Measures

Preclinical animal studies analysing the capacity of new technologies in cartilage regeneration frequently suffer from a lack of noninvasive follow-up and outcome measures and are therefore often forced to use endpoint outcome measures such as histology and destructive mechanical testing (Table 4.1). Additionally, there is an increasing need for standardized technologies with a diagnostic significance over the whole defect and adjacent tissues, while incorporating reflections of costs, care, ethics and mimicking the clinical investigations in human clinical trials (353, 365).

For longitudinal *in vivo* studies, it is advised to assess the animal at baseline and at different time points. Depending on the animal, healthy joint status at the start of the study should be evaluated via diagnostic imaging modalities since variability in cartilage thickness, bone structure and the prevalence of naturally occurring cartilage defects and other lesions associated with OA can occur among species (354, 374-376). More specifically, spontaneously occurring cartilage lesions have been described in canine, equine and ageing ovine models (17, 353, 354). Canine and equine models should be screened for naturally occurring OA, since they can have lesions associated with OA or osteochondritis dissecans (17, 353). Noninvasive imaging of articular cartilage defects can be performed by MRI (377-379) or computed tomography arthrography (CTA) (376, 380, 381). CTA has been shown to be more accurate than MRI to detect cartilage defects in humans (376, 382). Hontoir *et al.* described CTA to be an accurate imaging method for detecting articular cartilage defects in the ovine stifle (376). Additionally, the same author compared the sensitivity and specificity of 3-Tesla (3-T) MRI and CTA to identify structural cartilage defects in the equine metacarpo/metatarsophalangeal joint. Hontoir and colleagues showed that CTA is superior to MRI due to its shorter

acquisition time, enhanced correlation to macroscopic assessment and its specificity and sensitivity in identifying articular cartilage defects, nonetheless MRI has the advantage to assess soft tissues and subchondral bone (380).

For the visualization of cartilage, diagnostic imaging techniques such as ultrasound, computed tomography (CT) and MRI can be used (310, 365). More recently, novel quantitative MRI and CT techniques are being adopted as outcome measures after cartilage repair (365, 379, 381). Compositional imaging MRI is being progressively applied to assess the biochemical composition of cartilage for the longitudinal follow-up of cartilage repair studies (366). More specifically, T2 mapping combined with delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) seems to be a good compositional imaging modality to monitor cartilage repair and to discriminate between a collagen network with zonal organization and healthy cartilage (366, 383). Combining multiple imaging techniques may yield a better understanding of both the collagen and proteoglycan (PG) content of the repaired defect (384). T2 mapping provides information about the interaction of water molecules and the collagen network, while dGEMRIC evaluates glycosaminoglycan (GAG) concentration within cartilage (385). In human patients, Kurkijärvi *et al.* demonstrated that combining datasets from dGEMRIC and T2 relaxation time mapping provides additional information on cartilage repair (383). Recently, T2 mapping and dGEMRIC were used for assessing cartilage repair after allograft chondrocyte implantation in a rabbit model, where dGEMRIC data showed a high correlation with histological and biochemical data (385). In goat models, T2 mapping and dGEMRIC have also been used as outcome measures in a study evaluating cartilage repair after microfracture in an osteochondral defect of both the medial and lateral femoral condyles (386). One of the major disadvantages of using dGEMRIC is the necessity of administrating an intravenous contrast material (387). Alternatively, T1ρ has been used as a complementary imaging tool to T2 mapping which allows for the examination of PGs and the collagen organization and does not require the administration of a contrast agent (366, 387). Moreover, it offers information on early degenerative hallmarks and might offer prognostic values at baseline (387). Additionally, compared to T2 mapping, T1ρ might correlate better with macroscopic and histological characteristics of knee cartilage (388). However, one of the major issues of using T1ρ is reaching an adequate resolution with an

acceptable acquisition time and T1ρ is reported to be nonspecific in terms of cartilage components (366, 387). More recently, Van Tiel and colleagues showed that dGEMRIC is more robust in accurately measuring cartilage GAGs in vivo in patients compared to T1ρ mapping (389).

Although substantial progress has been made in real-time *in vivo* cartilage imaging, spatiotemporal tracking of stem cells *in vivo* using MRI, bioluminescence imaging (BLI), fluorescence imaging (FLI) or nuclear imaging methods, should be the focus when developing novel imaging techniques (365). Superparamagnetic iron oxide (SPIO) particles are used for cartilage tissue engineering to monitor transplanted cells (390, 391). However, SPIO particles are associated with several drawbacks such as the inability to distinguish viable cells from dead cells and from cells engulfed by phagocytes (392). One of the possibilities to minimize particle transfer to other cells is the use of reporter genes. BLI compatible reporter genes such as red/green luciferases have already been used for cartilage tissue engineering to track transplanted cells (393). In addition, by labelling cells with an additional chondrogenic reporter gene, cell differentiation can be monitored by means of dual bioluminescence labelling (394). While this optical imaging method offers a sensitive technique to track stem cells, its use in larger animal models is limited because of a loss of signal intensity from deeper tissues due to scattering (395).

At baseline and at longitudinal intervals, clinically relevant examinations of cartilage repair and functional improvement should be carried out. These should be performed by a veterinary surgeon familiar with observing clinical signs and locomotion by assessment of changes in joint palpation, quantitative monitoring of pain and changes in joint function or locomotion by gait analysis (310, 353, 396-399). In rats, several scoring systems have been published to measure lameness, stride length and limb rotation, dynamic force application and hind limb motion (399). Moreover, for large animal models, kinematic marker analysis, ground reaction force measurements, and observational gait assessment have been progressively used in OA-related gait alterations in canine, ovine and equine models (399). Several scaling systems have been documented in the literature, such as the American Association of Equine Practitioners (AAEP) lameness scale in the horse ranging from zero to five (400). In ovine models, a numeric ranking

scale can be used to determine comfort, movement and flock behaviour (397). A more detailed lameness scoring system has been published by Kaler *et al.* ranging from 'normal' (0) to 'unable to stand or move' (6) (396). Overall, clinical assessment and gait monitoring are indispensable in order to increase the translational value of preclinical animal studies to human clinical trials and to the clinic.

Biomarkers represent an additional tool to evaluate normal and pathological processes or to evaluate the interventional repair strategies (401, 402). These biomarkers may be identified and quantified via enzyme-linked immunosorbent assays (ELISA) or other protein assays in synovial fluid or other biological fluids such as in the blood and urine (401, 402). Synovial and other biological fluid collections should be performed at baseline and multiple time points (353), since synovial fluid biomarkers have the capacity to reflect the articular environment before treatment and could possibly inform on postoperative outcomes (401). In small animal models, however, it can be difficult to obtain sufficient amounts of biological fluid at multiple time points necessary for biomarker analysis (403). To solve this, the use of paper or alginate to obtain small amounts of synovial fluid has been described to be successful and effective (404). Because of the relatively larger joint size in large animal models, collection of synovial fluid and serum biomarkers can be more easily performed (347). Nevertheless, a major difficulty to perform repeated collections is the increased inflammation in the joint due to iatrogenic damage. Biomarkers of particular interest are markers for cartilage or synovium metabolism or markers involved in pathological pathways, such as inflammation (402). Recently, biological (synovial) fluid markers in OA were thoroughly reviewed by Nguyen and colleagues (402). Besides analyte quantifications to assess changes in inflammation and cartilage turnover, volume and physical characteristics of the synovial fluid, such as viscosity, could also be used as an outcome measure in preclinical studies (353).

At the end of *in vivo* studies, cadaver tissue can undergo *ex vivo* high resolution MRI (405, 406) and μCT (407) to evaluate structural improvements. Hereafter, macroscopic/arthroscopic scoring, histological and histomorphometric scoring methods, quantification of collagen and GAG expression by immunohistochemistry (IHC), collagen organization by polarized light microscopy and subchondral bone

and adjacent tissue integration are all outcome methods that should ideally be performed (407-411).

Nowadays, many histological scoring systems are available, contributing to the confusion on the use of an appropriate scoring method for a specific research question and study settings (412). Moreover, it is unclear which scoring systems are validated and how study results can be compared between studies using different scoring methods (412). The variety of histological scoring systems for analysis of normal or OA, *in vivo* repaired or *in vitro* tissue-engineered cartilage was thoroughly reviewed by Rutgers *et al*. (412). Normal cartilage can be distinguished from OA cartilage via the Histological-Histochemical Grading System (HHGS) or HHGS-related systems and the Osteoarthritis Research Society International (OARSI) scoring method (412). Of the various scoring systems available for analysis of *in* vivo repaired cartilage, the ICRS II score seems most suitable in humans. In preclinical cartilage repair studies, the validated Pineda score or O'Driscoll score is advisable (412). Other histological scoring systems for preclinical cartilage repair are widely used. In addition to the Pineda Score, the Wakitani score is an elementary scoring system, reflecting not more than five parameters (413). The Pineda score assesses four histological parameters: cell morphology, matrix staining, lesion filling and osteochondral junction (413). The O'Driscoll score is a more complex histological scoring method which also assesses surface regularity, structural integrity, cellularity, chondrocyte clustering, adjacent bonding, and adjacent cartilage degeneration. In addition to the O'Driscoll score, also the Fortier and Sellers scores are more comprehensive scoring systems (413). Orth *et al.* showed that both elementary and comprehensive histological scoring systems are appropriate to quantify articular cartilage repair (413). However, complex scoring systems provide more descriptive data about the character of the repair tissue (413). The use of validated scores, such as the Pineda Score or the O'Driscoll score, may significantly increase comparability of information and should thus stimulate consistency between studies. Importantly, histological and biochemical evaluations are complementary tools to assess experimental articular cartilage repair in vivo (412). A key goal of regenerating mature cartilage tissue is to regenerate a tissue with biochemical/biomolecular and mechanical properties resembling those of native cartilage tissue. Small biopsies for biochemistry (water

content, GAGs/PG content and collagen content) and/or biomechanical testing should ideally be gathered before fixation of the repaired tissue for histology (410). In addition to typical end-point destructive measures to assess mechanical properties, indentation testing provides a non-destructive compressive technique for *in situ* mechanical evaluation (365, 414). Large animal models allow the harvest of a large amount of repaired tissue in order to have parallel histological, biochemical and biomechanical analyses of the repaired area post-mortem (353, 415).

Finally, the combined utilization of *in vivo* clinical tests and assessment of locomotion, *in vivo* noninvasive imaging methods, and post-mortem evaluation of tissue structure with validated scoring systems, biochemical composition, and mechanical properties will deliver a robust outcome analysis in order to improve the translational value of animal models in cartilage repair.

4.5. Conclusion

Under ideal circumstances novel therapies are approved and released on the market after *in vitro* data were used to inform preclinical studies, which in turn lead to human clinical trials. Researchers should be aware that every animal model is associated with its advantages and disadvantages and the choice of model should match the research hypothesis and it is important to ensure proper translation to the clinic. Furthermore, the current lack of standardized protocols (i.e. cell delivery route, number of transplanted cells) as well as the wide variety of different outcome measures used to evaluate preclinical studies make it difficult to draw definite conclusions regarding the potential use of stem cell-based approaches in cartilage tissue engineering through direct comparison of studies. Furthermore, gender differences in most animal studies have not been adequately investigated and should gain more attention.

Musculoskeletal disorders (MSDs) include more than 150 diverse pathologies since they can affect but are not limited to muscles, bones, joints, cartilage, ligaments, and tendons. According to the World Health Organization (WHO), MSDs are the main cause for severe long-term pain and physical disability, and affect hundreds of millions of people around the world. Degeneration of the joint leads to injury to tissues from the joint, including articular cartilage and tendons. Cartilage injuries are very common, and form a risk factor for the development of osteoarthritis (OA), which is a degenerative and inflammatory condition of synovial joints with irreversible loss of supportive cartilage matrix. In addition to OA, over 30 million tendon-related surgeries take place per year worldwide with a significant socio-economic burden. Moreover, tendon lesions represent 30% of all musculoskeletal consultations. Unfortunately, both OA and tendinopathy involve tissues that are characterized by a low intrinsic healing capacity and current treatment options are not able to provide full and stable recovery of the damaged tissue. Therefore, there is a growing need for the development of new treatment options for OA, cartilage defects and tendon injuries. Autologous chondrocyte implantation or the transplantation of tendon-derived stem cells have been proposed as efficient cell-based therapies for treating chondral lesions or OA and tendon injuries respectively. However, the usage of adult autologous tissue-specific cells requires a two-step surgery and is associated with several other drawbacks. Therefore, innovative tissue engineering techniques exploiting compatible scaffolds and stem cells are currently needed. Stem cell-based therapies are seen as one of the most promising treatment strategies within regenerative research, since they have been widely used as therapeutic applications for many untreatable injuries and diseases.

For stem cell-based strategies for OA or tendinopathies, mesenchymal stem cells (MSCs) are of particular interest. Most studies using a MSC-based cell therapy focus on bone marrow-derived MSCs (BM-MSCs). However, this type of MSC is associated with several downsides. First, the isolation of BM-MSCs is invasive and is associated with several complications. Secondly, BM-MSCs often result in hypertrophic differentiation. A promising alternative stem cell niche can be found in tooth-associated tissues, such as the dental pulp or the periodontal ligament tissue. Dental pulp stem cells (DPSCs) are isolated from the dental pulp and were first isolated by Gronthos *et al.* Since their first isolation, several others revealed

their MSC-like characteristics, including their immunophenotyping, plastic adherence and the ability to differentiate into classical mesodermal cell lineages; adipocytes, osteocytes and chondrocytes *in vitro*. In contrast to BM-MSCs, DPSCs showed a higher proliferative rate and have an easy isolation procedure by which they can be obtained. Additionally, the immunomodulatory properties of DPSCs emphasize their promise as cell-based therapies for immune-related diseases. Periodontal ligament stem cells (PDLSCs) are isolated from the periodontal ligament tissue and have been described to be a promising cell source for tendon-regenerative applications because of their inherent ligamentous nature and their native expression pattern of tendon-associated markers.

In addition to MSCs, platelet concentrates are emerging as promising treatment possibilities because of their high amount in growth factors and cytokines, which have been described to play crucial roles in wound healing and immunomodulation. However, since the well-studied platelet derivative; platelet rich plasma (PRP), requires the supplementation of anti-coagulants and biochemical handling before preparation, the use of leukocyte- and platelet-rich fibrin (L-PRF), a second generation platelet concentrate, is encouraged.

In **Chapter 2** of the current dissertation, we evaluated the therapeutic application of DPSCs and L-PRF in OA via immunomodulation and cartilage regeneration. Strong paracrine-mediated effects of DPSCs in an *in vitro* OA model were shown, as they undergo chondrogenesis *in vitro*, stimulate the survival of chondrocytes and have immunomodulatory effects. In contrast, L-PRF did not show promising secretome-mediated effects on OA chondrocytes and was unable to enhance chondrogenesis of DPSCs and BM-MSCs *in vitro*.

In **Chapter 3**, we studied the ability of a three-dimensional (3D) growth condition under static tension and the supplementation of transforming growth factor-beta 3 (TGF-β3) to generate *in vitro* tendon-like tissues of DPSCs and PDLSCs and compared them to BM-MSCs. In this chapter, we validated the feasibility of the usage of PDLSCs as a novel cell source for tendon repair. Cell alignment, cell density and gel contraction seemed to be improved in PDLSC-seeded constructs. All three stem cell types showed positive expression of tendon-related markers, tenascin C and tenomodulin. In contrast to BM-MSCs and DPSCs, PDLSC-derived constructs displayed the presence of collagen fibrils and less bone and cartilage

components. Taken together, our study validated the usage of PDLSCs as a novel cell source for tendon repair

Before novel cell-based therapies for cartilage repair can be introduced into the clinic, rigorous testing in preclinical animal models is required. Preclinical models used in regenerative cartilage studies include murine, lapine, caprine, ovine, porcine, canine, and equine models, each associated with their specific advantages and limitations. **Chapter 4** represents an overview of the advantages and disadvantages of utilizing small and large animals and different outcome measures to assess cartilage repair. Researchers should be aware that every animal model is associated with its drawbacks and the choice of model strongly depends on the research hypothesis to ensure the translation to the clinic. Furthermore, the current lack of standardized protocols as well as the wide variety of outcome measures used to evaluate preclinical studies make it difficult to draw definite conclusions regarding the potential use of stem cell-based approaches in cartilage tissue engineering through direct comparison of studies.

Finally, for preclinical animal studies, the usage of autologous MSCs is the ideal approach to avoid rejection. In **Chapter 5**, we demonstrated that ovine DPSCs were effectively isolated from dental pulp tissue and showed morphological, phenotypical and functional properties similar to those observed in their human counterparts. We showed that ovine DPSCs may have potential use in osteochondral engineering. Moreover, because of promising paracrine-mediated effects by human DPSCs on OA-mimicked chondrocytes *in vitro*, preclinical OA studies in the ovine model using ovine DPSCs are encouraged.

Nonetheless, the work in this thesis paves the way for preclinical studies that focus on DPSC-based stem cell therapies for OA. Future studies should aim to test their (autologous) efficacy in a large animal model of OA and focus on longitudinal follow-up with non-invasive imaging methods. Moreover, PDLSCs have been proposed to be a promising alternative source, when compared to DPSCs or BM-MSCs, for cell-based regenerative treatment for tendon repair. However, future studies should aim to test their effectiveness in larger translation models.

Samenvatting

Musculoskeletale aandoeningen omvatten meer dan 150 verschillende pathologieën aangezien ze spieren, botten, gewrichten, kraakbeen, ligamenten en pezen kunnen aantasten. Volgens de Wereldgezondheidsorganisatie zijn musculoskeletale aandoeningen de hoofdoorzaak van langdurige pijn en lichamelijke beperkingen en treffen honderden miljoenen mensen over de hele wereld. Degeneratie van het gewricht kan leiden tot letsels aan weefsels van het gewricht, waaronder gewrichtskraakbeen en pezen. Kraakbeenletsels komen erg vaak voor en vormen een groot risico voor de ontwikkeling van osteoartritis, een degeneratieve en inflammatoire aandoening van synoviale gewrichten met onomkeerbaar verlies van ondersteunende kraakbeenmatrix. Naast osteoartritis, vinden wereldwijd jaarlijks meer dan 30 miljoen pees-gerelateerde ingrepen plaats met een aanzienlijke sociaaleconomische last. Bovendien vertegenwoordigen peeslaesies 30% van alle musculoskeletale consultaties. Helaas hebben zowel osteoartritis als tendinopathieën betrekking tot weefsels die worden gekenmerkt door een laag intrinsiek regeneratie vermogen en de huidige behandelingsopties zijn niet in staat om volledig en stabiel herstel van het beschadigde weefsel te verzekeren. Daarom is er een sterke nood aan de ontwikkeling van nieuwe behandelingsopties voor osteoartritis, kraakbeendefecten en peesblessures. Autologe chondrocytenimplantatie of transplantatie van pees-stamcellen werden reeds voorgesteld als efficiënte cel-gebaseerde therapieën voor de behandeling van respectievelijk chondrale laesies of osteoartritis en peesletsels. Het gebruik van autologe weefselspecifieke cellen vereist echter een tweestaps operatie en gaat gepaard met verschillende andere nadelen. Daarom zijn momenteel innovatieve weefseltechnieken nodig die gebruik maken van compatibele scaffolds en stamcellen. Stamcel-gebaseerde therapieën worden gezien als een van de meest veelbelovende behandelingsstrategieën binnen regeneratief onderzoek, aangezien ze voor veel onbehandelbare pathologieën op grote schaal onderzocht en gebruikt worden.

Voor stamcel-gebaseerde behandelingsstrategieën voor osteoartritis of tendinopathieën bieden mesenchymale stamcellen (MSCs) bijzondere voordelen. De meeste MSC-gebaseerde studies richten zich vooral op beenmerg MSCs (BM-MSCs). Dit type MSC gaat echter gepaard met verschillende nadelen. Ten eerste is de isolatie van BM-MSCs erg invasief en kan gepaard gaan met verschillende complicaties. Ten tweede resulteren BM-MSCs vaak in hypertrofische

differentiatie. Een veelbelovend alternatieve niche voor stamcellen zijn tand-geassocieerde weefsels, zoals de tandpulp of het parodontale ligamentweefsel. Dentale pulpa stamcellen (DPSCs) worden geïsoleerd uit de tandpulp en werden voor het eerst geïsoleerd door Gronthos *et al*. Sinds hun eerste isolatie onthulden verschillende anderen hun MSC-achtige kenmerken, waaronder hun immunofenotypering, vermogen om te hechten aan plastiek en het vermogen om te differentiëren naar klassieke mesodermale cellijnen; adipocyten, osteocyten en chondrocyten *in vitro*. In tegenstelling tot BM-MSCs, vertoonden DPSCs een hogere proliferatief vermogen en hebben ze een gemakkelijke isolatieprocedure waarmee ze kunnen worden verkregen. Bovendien benadrukken de immunomodulerende eigenschappen van DPSCs hun veelbelovend gebruik voor immuun-gerelateerde ziekten. Parodontale ligament stamcellen (PDLSCs) worden geïsoleerd uit het parodontale ligamentweefsel en werden reeds beschreven als een veelbelovende celbron voor peesregeneratieve toepassingen vanwege hun inherente ligamenteuze aard en hun natuurlijke expressiepatroon van pees-geassocieerde merkers.

Naast MSCs worden plaatjesconcentraten meer en meer als veelbelovende behandelingsmogelijkheden voorgesteld vanwege hun hoge concentratie aan groeifactoren en cytokines, waarvan is beschreven dat ze cruciale rollen spelen bij wondgenezing en immunomodulatie. Echter, aangezien het bekende bloedplaatjesderivaat; bloedplaatjesrijk plasma (PRP), de toevoeging van anti-coagulantia en biochemische behandeling vóór bereiding vereist, wordt het gebruik van leukocyten- en bloedplaatsjesrijk fibrine (L-PRF), een bloedplaatjesconcentraat van de tweede generatie, aangemoedigd.

In **Hoofdstuk 2** van het huidige doctoraatsproefschrift evalueerden we de therapeutische toepassing van DPSCs en L-PRF in osteoartritis via immunomodulatie en kraakbeenregeneratie. Sterke paracrien-gemedieerde effecten van DPSCs in een *in vitro* osteoartritis-model werden aangetoond, aangezien ze *in vitro* chondrogenese ondergaan, de overleving van chondrocyten stimuleren en immunomodulerende effecten hebben. Daarentegen vertoonde L-PRF geen veelbelovende secretoom-gemedieerde effecten op osteoartritis-chondrocyten en was L-PRF niet in staat de chondrogenese van DPSCs en BM-MSCs *in vitro* te versterken.

In **Hoofdstuk 3** bestudeerden we het vermogen van een driedimensionale celcultuur omgeving onder statische spanning en de toevoeging van transforming growth factor-beta 3 (TGF-β3) om *in vitro* peesachtige-constructen van DPSCs en PDLSCs te genereren en te vergelijken met BM-MSCs. In dit hoofdstuk hebben we het gebruik van PDLSCs als nieuwe celbron voor peesherstel gevalideerd. Celalignering, celdichtheid en gelcontractie waren sterker in PDLSC-afkomstige constructen. Alle drie stamceltypen vertoonden positieve expressie van pees-gerelateerde merkers, tenascin C en tenomoduline. In tegenstelling tot BM-MSCs en DPSCs, vertoonden PDLSC-afkomstige constructen de aanwezigheid van collageenfibrillen en minder bot- en kraakbeencomponenten. Tot slot, heeft onze studie het gebruik van PDLSCs als een nieuwe celbron voor peesherstel gevalideerd.

Vooraleer nieuwe cel-gebaseerde therapieën voor kraakbeenherstel in de kliniek kunnen worden geïntroduceerd, is rigoureus testen in preklinische diermodellen vereist. Preklinische modellen die worden gebruikt in regeneratieve kraakbeenstudies omvatten muizen, konijnen, geiten, schapen, varkens, honden en paarden, elk geassocieerd met hun specifieke voordelen en beperkingen. **Hoofdstuk 4** geeft een overzicht van de voor- en nadelen van het gebruik van kleine en grote diermodellen en de verschillende evaluatiemethoden om kraakbeenherstel te bestuderen. Onderzoekers moeten zich ervan bewust zijn dat elk diermodel geassocieerd kan worden met nadelen en dat de keuze van het model sterk afhangt van de onderzoekshypothese om de translatie naar de kliniek te verzekeren. Bovendien maken het huidige gebrek aan gestandaardiseerde protocollen en de grote verscheidenheid aan evaluatiemethoden het moeilijk om definitieve conclusies te trekken over het mogelijke gebruik van stamcel-gebaseerde behandelingsstrategieën voor kraakbeenweefsel door directe vergelijking van studies.

Voor preklinische dierstudies zou het gebruik van autologe MSCs de ideale strategie zijn om afstoting te voorkomen. In **Hoofdstuk 5** toonden we aan dat schaap DPSCs effectief geïsoleerd werden uit de dentale pulpa en morfologische, fenotypische en functionele eigenschappen vertoonden die gelijkaardig waren aan humane DPSCs. We toonden aan dat DPSCs van schapen mogelijk van belang zouden kunnen zijn voor osteochondrale engineering. Hierbij worden preklinische

osteoartritis-studies in het schapenmodel met schaap DPSCs aangemoedigd vanwege de veelbelovende paracrien-gemedieerde effecten van DPSCs in een osteoartritis model *in vitro*.

Desalniettemin effent het werk in dit doctoraatsproefschrift de weg naar preklinische studies die zich richten op DPSC-gebaseerde stamceltherapieën voor osteoartritis. Vervolgstudies dienen zich te richten op het testen van hun (autologe) werkzaamheid in een groot diermodel van osteoartritis en zich richten op een longitudinale follow-up met niet-invasieve beeldvormingsmethoden. Bovendien werden PDLSCs als een veelbelovende alternatieve bron voorgesteld voor cel-gebaseerde regeneratieve behandelingen voor peesherstel. Toekomstige studies moeten er echter op gericht zijn hun doeltreffendheid in grotere diermodellen te testen.

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Curriculum Vitae

Melissa Lo Monaco was born on June 18th, 1993 in Taormina, Italy. She finished the secondary education in 2011 (Greek-Mathematics) at Onze-Lieve-Vrouwlyceum Genk. She continued her studies at Hasselt University (Diepenbeek, Belgium), where she graduated as a Bachelor (2011-2014) with satisfaction and Master (2014-2016) with magna cum laude in Biomedical Sciences. Her master thesis was entitled "Human dental pulp stem cells as a cell-based therapy to target angiogenesis in head and neck cancer" and was achieved at the Laboratory of Morphology of the Biomedical Research Institute of Hasselt University under the supervision of Prof. dr. Ivo Lambrichts and Prof. dr. Annelies Bronckaers. In October 2016, she started her PhD in the laboratory of Morphology of the Biomedical Research Institute of Hasselt University under the supervision of Prof. dr. Ivo Lambrichts and at the Integrated Veterinary Research Unit of the University of Namur under the supervision of Prof. dr. Jean-Michel Vandeweerd. The research that is described in this thesis was conducted between October 2016 and July 2020. During this period, the PhD programme of The Doctoral Schools of Medicine and Life Sciences of Hasselt University and the Doctoral Training of "Santé, Sciences et Techniques" of the University of Namur were successfully completed.

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Poster and oral presentations

Posters (Presenting Author only)

Lo Monaco M. The potential applications of dental stem cells for cell-based therapy. Narilis Research Day. 17 November 2017, Namur, Belgium

Lo Monaco M, Gervois P, Clegg P, Bronckaers A, Vandeweerd JM* & Lambrichts I*. Dental pulp stem cells and leukocyte- and platelet-rich fibrin (L-PRF) for articular cartilage repair. 4th Annual Meeting of the Belgian Society for Stem Cell Research (BESSCR). 24 November 2017, Liège, Belgium

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Lo Monaco M. Dental pulp stem cells and leukocyte- and platelet-rich fibrin for articular cartilage repair. PhD Student Day. 19 May 2019, Brussels, Belgium

Lo Monaco M, Gervois P, Beaumont J, Clegg P, Bronckaers A, Vandeweerd JM* & Lambrichts I*. Dental pulp stem cells and leukocyte- and platelet-rich fibrin for articular cartilage repair. TERMIS EU. 27-32 May 2019, Rhodes, Greece

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Oral Presentation

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